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Valorization of distillery stillage by recovering polyphenolic compounds

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Waloryzacja wywaru gorzelniczego przez odzysk związków polifenolowych

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List of thematically coherent publications constituting the basis of the Ph.D. Thesis

The Ph.D. thesis consists of five peer-reviewed scientific publications that have been published in the journals included in the list of scientific journals (according to the announcement of the Minister of Education and Science on 21.12.2021), one manuscript that has been accepted for publication and one manuscript that has been submitted to the journal.

P1. **Mikucka Wioleta,** Zielińska Magdalena, 2020. Distillery stillage: characteristics, treatment, and valorization. *Applied Biochemistry and Biotechnology* 192(3), 770–793, https://doi.org/10.1007/s12010-020-03343-5. **(IF= 2.926; 70 points)**

P2. **Mikucka Wioleta,** Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela, 2022. Recovery of polyphenols from distillery stillage by microwave-assisted, ultrasound-assisted and conventional solid-liquid extraction. *Scientific Reports* 12, 3232, https://doi.org/10.1038/s41598-022-07322-0. **(IF= 4.996; 140 points)**

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P4. **Mikucka Wioleta,** Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela. Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions. (To *Journal of Environmental Management*; manuscript ref: JEMA-D-22-05107R1; submission date: 10.06.2022; current status: accepted for publication) **(IF= 8.910; 100 points)**.

P5. **Mikucka Wioleta**, Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela, 2022. Subcritical water extraction of bioactive phenolic compounds from distillery stillage. *Journal of Environmental Management* 318, 115548, https://doi.org/10.1016/j.jenvman.2022.115548. **(IF= 8.910; 100 points)**

P6. **Mikucka Wioleta**, Zielinska Magdalena, 2022. Individual phenolic acids in distillery stillage inhibit its biomethanization. *Energies* 15, 5377, https://doi.org/10.3390/en15155377. **(IF= 3.252; 140 points)**

P7. **Mikucka Wioleta**, Witonska Izabela, Zielinska Magdalena, Bulkowska Katarzyna, Binczarski Michał. The concept for the circular economy in an ethanol production plant. (To *Chemosphere*; manuscript ref: CHEM108686; submission date: 28.08.2022; current status: submitted to the journal) **(IF= 8.943; 140 points)**.

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Attachments – publications constituting the Ph.D. Thesis:

Attachment 1.

P1. Mikucka Wioleta, Zielińska Magdalena, 2020. Distillery stillage: characteristics, treatment and valorization. *Applied Biochemistry and Biotechnology* 192(3), 770–793.

Attachment 2.

P2. Mikucka Wioleta, Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela, 2022. Recovery of polyphenols from distillery stillage by microwave-assisted, ultrasound-assisted and conventional solid-liquid extraction. *Scientific Reports* 12, 3232.

Attachment 3.

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Attachment 4.

P4. Mikucka Wioleta, Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela. Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions. Accepted for publication in *Journal of Environmental Management*.

Attachment 5.

P5. Mikucka Wioleta, Zielinska Magdalena, Bulkowska Katarzyna, Witonska Izabela, 2022. Subcritical water extraction of bioactive phenolic compounds from distillery stillage. *Journal of Environmental Management* 318, 115548.

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Attachment 7.

P7. Mikucka Wioleta, Witonska Izabela, Zielinska Magdalena, Bulkowska Katarzyna, Binczarski Michał. The concept for the circular economy in an ethanol production plant. Submitted to *Chemosphere*.

Abstract

In the thesis, a valorization concept consisting of the multidirectional use of waste biomass from distilleries was proposed, which is commensurate with the circular economy principles in which waste is used as a raw material. This strategy allows for contributing to the sustainable development of the distillery industry and diminishing the environmental pollution resulting from distillery operations.

In this study, the recovery of bioactive compounds was proposed as an option for the valorization of distillery stillage coming from cereal processing to alcohol. Because the content of extracted polyphenols and the resulting antioxidant activity of the product depend on the substrate used for alcohol production and the method of recovery, there is a necessity to optimize extracting conditions for each waste material. Therefore, the objective of this study was to determine the potential of distillery stillage as a source of bioactive polyphenols, the possibilities of their recovery, and the effect of phenolic acids on methane production from distillery stillage. The studies included: i) extraction of polyphenols from the stillage with commercial organic solvents by using conventional solid-liquid extraction (CSLE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), and with water by using subcritical water extraction (SWE), ii) the use of tetrahydrofurfuryl alcohol (THFA) and biochar produced from the solid fraction of cereal processing waste (including distillery stillage) to recover phenolic acids from the liquid fraction of the stillage, and iii) investigating the effect of phenolic acids on the methane potential of distillery stillage.

It was shown that distillery stillage was a source of bioactive polyphenols. All the solvents used (ethanol, methanol, acetone, ethyl acetate, water) could be applied in the extraction of polyphenols from the stillage, but the yield and composition of polyphenols, extraction rate, and antioxidant activity as a measure of the extract quality depended on the type and concentration of the solvent and the method and conditions of extraction (solid-to-solvent ratio, time, temperature). Because of the more efficient recovery of polyphenols, higher antioxidant activity of the extracts and a significantly shorter time of extraction, MAE and UAE were found to be “green” alternatives to conventional extraction. Independently of the extraction technique, ethanol was the most effective solvent in recovering polyphenols. The use of SWE resulted in a high content of polyphenols in the extracts, with simultaneous elimination of organic solvents from the extraction process. The biomass remaining after SWE of polyphenols can be considered

a valuable product – hydrochar. However, the antioxidant activity of the extracts after SWE was a few times lower than those obtained with ethanol. The extracts that were characterized by the highest antioxidant activities resulted from MAE with 80% ethanol, 80% methanol and 70% ethyl acetate. The addition of water into all organic solvents increased the content of polyphenols and antioxidant activity of the extracts.

Synergistic processing of different streams of cereal waste may be a promising direction in the waste-to-profit management of agro-food waste. It was investigated that not only commercial solvents but also waste-derived THFA and biochar were suitable for the recovery of phenolic acids from distillery stillage. The recovery efficiency depended on THFA concentration, biochar dose, and desorption temperature. The content of phenolic acids in the THFA extracts was higher than those obtained with acetone, ethyl acetate and water, similar to that obtained with methanol, and lower by about 7% than that obtained with ethanol. Adsorption/desorption of phenolic acids from the extracts allowed to maintain ~95% of the antioxidant activity.

In the extracts obtained with all extraction conditions and solvent types, polyphenols that were efficient at scavenging radicals predominated over those with a reducing power. Independently of the extraction conditions, phenolic acids were present in the extracts mainly in the free form, which allows to avoid hydrolysis of the stillage before the extractions. Among these acids, ferulic and *p*-coumaric acids (both belonging to hydroxycinnamic acids) predominated. Such characteristics of the extracts open various possibilities for the industrial applications of recovered phenolic compounds.

The negative effect of phenolic acids on the methane potential of distillery stillage strongly depended on the type of acid and its concentration. The presence of phenolic acids decreased the abundance of acetoclastic and hydrogenotrophic methanogens during the stillage fermentation. The obtained results concerning the inhibition constants of methane production with particular phenolic acids may be valuable for improving biogas production from wastes with different compositions and concentrations of bioactive compounds.

Keywords: valorization of distillery stillage; recovery of bioactive compounds; phenolic antioxidants; distilleries by-products; methane potential; circular bioeconomy

Streszczenie

W pracy doktorskiej zaproponowano koncepcję waloryzacji polegającą na wielokierunkowym wykorzystaniu biomasy odpadowej z gorzelni, co jest zgodne z zasadami gospodarki o obiegu zamkniętym, w której odpady są wykorzystywane jako surowiec. Strategia ta pozwala przyczynić się do zrównoważonego rozwoju przemysłu gorzelniczego i zmniejszenia zanieczyszczenia środowiska wynikającego z działalności gorzelni.

Jako opcję waloryzacji wywaru gorzelniczego, w badaniach zaproponowano odzyskiwanie związków bioaktywnych z wywaru pochodzącego z przetwórstwa zbóż. Ponieważ zawartość ekstrahowanych polifenoli i aktywność antyoksydacyjna produktu zależą od substratu użytego do produkcji alkoholu oraz sposobu odzysku, istnieje konieczność optymalizacji warunków ekstrakcji dla każdego materiału odpadowego. Celem pracy było więc określenie potencjału wywaru gorzelniczego jako źródła bioaktywnych polifenoli, możliwości ich odzysku oraz wpływu kwasów fenolowych na produkcję metanu z wywaru gorzelniczego. Badania obejmowały: i) ekstrakcję polifenoli z wywaru za pomocą komercyjnych rozpuszczalników organicznych przy użyciu konwencjonalnej ekstrakcji ciało stałe-ciecz (CSLE), ekstrakcji wspomaganej ultradźwiękami (UAE) i ekstrakcji wspomaganej mikrofalami (MAE) oraz za pomocą ekstrakcji wodą w stanie podkrytycznym (SWE), ii) zastosowanie alkoholu tetrahydrofurfurylowego (THFA) i biowęgla wytwarzanych z frakcji stałej odpadów z przetwórstwa zbóż (w tym wywaru gorzelniczego) w celu odzyskiwania kwasów fenolowych z ciekłej frakcji wywaru gorzelniczego oraz iii) badanie wpływu kwasów fenolowych na potencjał metanowy wywaru gorzelniczego.

Wykazano, że wywar gorzelniczy był źródłem bioaktywnych polifenoli. Wszystkie użyte rozpuszczalniki (etanol, metanol, aceton, octan etylu, woda) mogły być stosowane w ekstrakcji polifenoli z wywaru, ale wydajność i skład polifenoli, szybkość ekstrakcji i aktywność antyoksydacyjna jako miara jakości ekstraktu zależały od rodzaju i stężenia rozpuszczalnika oraz metody i warunków ekstrakcji (stosunek cząstek stałych do rozpuszczalnika, czas, temperatura). Ze względu na bardziej efektywny odzysk polifenoli, wyższą aktywność antyoksydacyjną ekstraktów i znacznie krótszy czas ekstrakcji, MAE i UAE okazały się "zielonymi" alternatywami dla konwencjonalnej ekstrakcji. Niezależnie od techniki, etanol był najskuteczniejszym rozpuszczalnikiem w ekstrakcji polifenoli. Zastosowanie SWE skutkowało wysoką zawartością polifenoli

w ekstraktach, przy jednoczesnej eliminacji rozpuszczalników organicznych z procesu ekstrakcji. Biomasę pozostałą po SWE można uznać za cenny produkt – hydrowęgiel. Aktywność antyoksydacyjna ekstraktów uzyskanych w SWE była jednak kilkakrotnie niższa niż ekstraktów otrzymywanych w ekstrakcji z etanolem. Ekstrakty o najwyższej aktywności antyoksydacyjnej pochodziły z MAE z wykorzystaniem 80% etanolu, 80% metanolu i 70% octanu etylu. Dodanie wody do wszystkich rozpuszczalników organicznych zwiększyło zawartość polifenoli i aktywność antyoksydacyjną ekstraktów.

Synergiczne przetwarzanie różnych strumieni odpadów z przetwórstwa zbóż może być obiecującym kierunkiem w gospodarowaniu tymi odpadami. Wykazano, że nie tylko komercyjne rozpuszczalniki, ale także produkowane z odpadów THFA i biowęgiel mogą być wykorzystywane do odzyskiwania kwasów fenolowych z wywaru gorzelniczego. Skuteczność odzysku zależała od stężenia THFA, dawki biowęgla i temperatury desorpcji. Zawartość kwasów fenolowych w ekstraktach z THFA była wyższa niż w ekstraktach otrzymywanych podczas ekstrakcji z acetonem, octanem etylu i wodą, podobna do uzyskiwanej podczas ekstrakcji z metanolem i niższa o około 7% od otrzymywanej w ekstrakcji z etanolem. Adsorpcja/desorpcja kwasów fenolowych z ekstraktów pozwoliła na utrzymanie ~95% aktywności antyoksydacyjnej.

Niezależnie od warunków ekstrakcji, uzyskane polifenole, które były skuteczne w usuwaniu rodników, przeważały nad tymi o mocy redukcyjnej. Kwasy fenolowe występowały w ekstraktach głównie w postaci wolnej, co pozwala uniknąć hydrolizy wywaru przed ekstrakcjami. Wśród wyekstrahowanych kwasów przeważały kwasy ferulowy i *p*-kumarowy (oba należące do kwasów hydroksycynamonowych). Taka charakterystyka ekstraktów otwiera różne możliwości przemysłowych zastosowań uzyskanych związków fenolowych.

Negatywny wpływ kwasów fenolowych na potencjał metanowy wywaru gorzelniczego silnie zależał od rodzaju i stężenia kwasu. Obecność kwasów fenolowych zmniejszała liczebność metanogenów acetoklastycznych i wodorotroficznych podczas fermentacji wywaru. Wyniki dotyczące stałych inhibicji produkcji metanu w obecności poszczególnych kwasów fenolowych mogą być wykorzystywane do poprawy produkcji biogazu z odpadów o różnym składzie i stężeniu związków bioaktywnych.

Słowa kluczowe: waloryzacja wywaru gorzelniczego; odzysk związków bioaktywnych; przeciwutleniacze fenolowe; produkty uboczne gorzelni; potencjał metanowy; biogospodarka o obiegu zamkniętym

1. Introduction to the subject of research

The distillery industry is a significant element of the global economy but is water-intensive, energy-intensive, and generates huge amounts of by-products. One of these by-products is whole stillage (named distillery stillage) that remains after the production of ethanol from cereal grains. It is a liquid that contains, depending on the production technology, 7–20% of dry weight, which includes proteins, fibers, unhydrolyzed starch, fat and dead yeast cells (Mikulski and Kłosowski 2018). As a result of the production of 1 liter of alcohol, up to 15 liters of distillery stillage is generated (Fito et al. 2019). Thus, a standard-issue distillery can produce about 150,000 liters of alcohol per day while generating 1,950,000 liters of the stillage (Gebreeyessus et al. 2019). Distillery stillage is a complex waste that pollutes the environment due to its acidic nature (3.0–4.5 pH), dark brown color (due to the presence of melanoidins), high concentration of organic compounds (110,000–190,000 mg COD/L, 50,000–60,000 mg BOD/L), heavy metals and macro- and microminerals (Fito et al. 2019, Ratna et al. 2021). Melanoidins are created during a non-enzymatic Maillard reaction of reducing sugar and amine compounds at high temperatures (Cooper et al. 2019). When released into the environment, melanoidins block the penetration of sunlight in water bodies, thus contributing to inhibition of photosynthesis and a reduction in dissolved oxygen concentration, which negatively affects aquatic organisms (Espana-Gamboa et al. 2011). In addition, distillery waste can cause water eutrophication due to its high levels of nutrients (Kharayat 2012). To reduce the environmental impact of the stillage, biological (aerobic and anaerobic), physicochemical (coagulation/flocculation, electrocoagulation, adsorption, advanced oxidation and membrane processes) and thermal (evaporation/combustion) methods were tested (Ghosh et al. 2004). To fully purify the by-products of the distillery, especially in terms of water reuse, a comprehensive treatment should be applied that includes several successive technologies.

The distillery stillage is used as feed for farm animals (Klopfenstein et al. 2008), field fertilizer (Fito et al. 2019), or for biogas production (Caruso et al. 2019), which requires storage before seasonal applications, thus releasing methane, ammonia and nitrous oxide. In addition, effective use of the stillage as fodder requires separation of the whole stillage into a solid and liquid fraction and drying the solid fraction to increase the content of dry weight, thus increasing the calorific value (Mikulski and Kłosowski 2018). Otherwise, the use of stillage as fodder is not effective. Therefore, due to the increasing

demand for alcohol production, particularly for medical and fuel purposes (Cooper et al. 2019, Klemeš et al. 2020), it is important to develop the strategies of stillage management to promote the circular economy and to valorize this waste to a greater extent. This concept includes recovering compounds with valuable functions.

For alcohol production, cereal grains, potato starch, sugar beet and fruit molasses are used (Smuga-Kogut 2015). Plant-origin raw materials used for alcohol production contain bioactive compounds such as polyphenols that include flavonoids (e.g. flavanols, condensed tannins, anthocyanins or proanthocyanidins) and non-flavonoids (e.g. phenolic acids, lignins, stilbenes, ellagotanins and gallotannins) (Shahidi and Ambigaipalan 2015). The basic structure of flavonoids consists of a flavonoid nucleus containing 15 carbon atoms arranged in three rings (C6–C3–C6). Flavonoids exist mainly as glycosides and polymers. Phenolic acids are derivatives of benzoic and cinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechic, vanillic and syringic acid, which share a C6–C1 structure. In turn, hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C6–C3), most often represented by caffeic, ferulic, *p*-coumaric and sinapic acids (Clifford 1999, Manach et al. 2005). Phenolic acids exist in a free or bound form or as free esters. They are found combined with structural components of the cell wall such as cellulose, lignin, tannins and proteins. Under acidic conditions, these bonded structures can undergo hydrolysis (Liu 2007). In addition, industrial processes such as heat treatment, pasteurization, fermentation and freezing contribute to the release of phenolic compounds (Dewanto et al. 2002). Therefore, the approach using alcohol production waste as a rich and cheap source of bioactive compounds may be of particular interest. However, due to the antimicrobial activity, polyphenols at concentrations above 1 g/m^3 may reduce the ability of methanogens to produce methane (Hernandez and Edyvean 2008). On the other hand, the presence of polyphenols in distillery stillage can be considered in a positive light. Due to their antioxidant, antibacterial, antiviral, anticancer and anti-inflammatory properties (Shahidi and Ambigaipalan 2015), polyphenols have gained much attention as valuable bioactive compounds of commercial importance, to be used in the food, pharmaceutical and cosmetic industries (Kalogerakis et al. 2013). They can replace synthetic antioxidants used in food products, such as butylated hydroxyanisole, butylated hydroxytoluene, tert-butylhydroquinone or propyl gallate, which can be toxic and carcinogenic (Shahidi and Ambigaipalan et al. 2015). Therefore, this approach will not only reduce the pollution generated during alcohol production but also recover valuable products, thus improving the anaerobic treatment of

the waste (Kaushik et al. 2018). This can promote the viability of one of the most commonly used methods of distillery stillage valorization (biogas production), thus contributing to the economic competitiveness and sustainable development of the distillery industry (Tapia-Quirós et al. 2022a).

The antioxidant activity of polyphenols depends on their ability to scavenge free radicals, disrupt radical chain reactions and chelate metals. Due to the variety of these reactions, the antioxidant activity can be monitored by several methods, which include the use of ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) assays. ABTS test is based on hydrogen atom transfer (HAT), whereas DPPH and FRAP are based on single electron transfer (SET) (Awika et al. 2003). HAT is a one-step reaction that depends on the enthalpy of hydroxyl bond dissociation, while SET is a two-step reaction that depends on the ionization potential and proton dissociation enthalpy, and proton affinity and electron transfer enthalpy (Mazzone et al. 2015). These reaction mechanisms can occur independently or simultaneously at different rates depending on the raw material and the environmental conditions in which the bioactive compounds occur (Shadnia and Wright 2008). Moreover, the action of antioxidants is strongly related to their structure. In the case of phenolic acids, the antioxidant activity is directly influenced by the phenolic hydroxyl, methoxy and carboxyl groups. Depending on the raw material and the conditions of polyphenol recovery, the activity also depends on intermolecular interactions between different bioactive compounds, which may be antagonistic or synergistic (Luis et al. 2018). Therefore, to estimate the antiradical capacity or the reducing power of recovered polyphenols and to demonstrate the relationship between the structure of polyphenols from the newly tested material (e.g. stillage) and their antioxidant activity, it is advisable to conduct several tests to comprehensively determine the antioxidant activity of the recovered bioactive compounds.

Since polyphenols differ in their chemical structure, interactions with other components and the strength of the association with the matrix (Guido and Moreira 2017), optimizing the recovery conditions is a very important aspect. The most popular techniques of recovery are liquid-liquid extractions and solid phase extractions (Khezeli et al. 2016). The organic solvents used to recover polyphenols are acetone, ethyl acetate, methanol, ethanol, and propanol or mixtures thereof. Different solvents extract different polyphenols because of affinity differences resulting from distinct polarities of a solvent and a target compound (Chaves et al. 2020). For example, when comparing acetone-water

and ethanol-water solvents of concentrations 0–100% for extracting phenolic compounds from brewers' spent grain, 60% acetone was indicated the optimal (Zuorro et al. 2019). From the range of solvents (40–100% methanol, 40–100% ethanol, 40–100% acetone, 100% heksane, 100% ethyl acetate and water), the use of 100% methanol, 60% ethanol, and 60% acetone resulted in the highest phenolic content (Socaci et al. 2018). For the same waste material, 60 and 80% ethanol gave the highest phenolic content; however, the properties of the extracts differed significantly in the range of their biological activities (Bonifacio-Lopes et al. 2020). When extracting phenolic compounds from buckwheat, extracts obtained with the use of 80% acetone, 100% ethyl acetate, and 70% ethanol differed significantly in terms of the composition of the extracted polyphenols (Chen et al. 2020). In addition, the affinity of the target compound to the solvent can be changed by interactions between the bioactive compounds and other components of the substrate (Chaves et al. 2020). Polar solvents are usually considered better than non-polar solvents; therefore, the modification of solvents improves extraction efficiency (Xie et al. 2019). Thus, to increase the solvation power and the recovery yield of polyphenols, different combinations of solvents and their mixtures should be investigated.

As an alternative to commercial solvents, biomass-derived solvents (named eutectic solvents) such as ethyl lactate, glycerol or 2-methyltetrahydrofuran can be used. These solvents are renewable, non-toxic and not very expensive, but their scope of application and solvation properties are limited (Ruesgas-Ramon et al. 2017). Bio-solvents can be produced from renewable biomass sources such as energy crops, forest products, aquatic biomass and waste materials from the sugar industry, distilleries or mills (Torres-Valenzuela et al. 2020). Cereal processing, which is a source of distillery waste itself, may be also a source of natural solvents. During biomass hydrolysis, pentosans are formed which are then catalytically converted into furfural. Furfural is the so-called platform compound, which means that it is a renewable building block for products with great market potential. Furfural can be catalytically hydrogenated to e.g. tetrahydrofurfuryl alcohol (THFA) (Modelska et al. 2017). THFA may be a promising and ecological alternative to conventional organic solvents due to its miscibility with water, low volatility and good thermal stability (Skrollahzadeh et al. 2008); it has been approved by the Environmental Protection Agency as a natural and biodegradable solvent safe for the environment. So far, THFA has not been used for the extraction of phenolic compounds from distillery stillage. The use of waste-derived solvents for the extraction of bioactive compounds may generate economic benefits and positively affect the

disposal of industrial by-products. However, because the extraction yield is affected by the affinity between the solvent and the target compound and the extraction conditions, the use of a solvent that is not recognized to extract a particular target compound requires optimization.

Although the recovery of polyphenols from waste materials may employ the conventional solid-liquid extraction (CSLE) due to its simplicity and low cost (Drużyńska et al. 2017), to meet the requirements of sustainable development, the recovery of polyphenols should be carried out using "green" extraction techniques that minimize the volume of solvents and reduce the extraction time (Chemat et al. 2012). Therefore, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and subcritical water extraction (SWE) have been developed (Barba et al. 2016). Both MAE, based on rapid heating of the solvent by electromagnetic energy (causing molecular movement through ionic conductivity and dipole rotation), and UAE, based on acoustic cavitation, increase the penetration of the solvent into the substrate, improving the mass transfer rate. MAE and UAE offer the following advantages over CSLE: they can reduce the consumption of organic solvents and can shorten the extraction time (Vinatoru et al. 2017). For example, the higher the dielectric constant or the more polar the solvent, the greater its ability to absorb microwaves, thus improving the mass transfer rate, which increases the solvation of the extracted compounds. The SWE technique proves to be an environmentally friendly alternative because it uses solvents at temperatures above their atmospheric boiling point and the elevated pressure keeps the solvent liquid (Attard and Hunt 2018). Under these conditions, the dielectric constant and polarity of water decrease (Teo et al. 2010). As a result, water has the properties of organic solvents, which is highly suitable for the extraction of phenolic compounds. Under SWE conditions, water shows hydrolytic properties, which allows the release of bound compounds and the breakdown of larger molecules while maintaining high biological and antioxidant activity (Plaza and Turner 2015). However, these extraction techniques do not guarantee better extraction results, especially in the case of polyphenols which are thermolabile compounds (Moreira et al. 2012). Taking the above into consideration, due to the different chemical and physical properties of target compounds and solvents, it is necessary to optimize the extraction conditions for every solvent to maximize the recovery yield of polyphenols and the antioxidant activity of the extracts.

From a practical point of view, the purification and concentration of the product obtained after extraction are crucial. One of the most common methods is adsorption on

activated carbon, which is used in many industrial sectors due to the low cost and simplicity of the process. The high specific surface area and microporosity of activated carbon make it ideal for the selective recovery of bioactive compounds (Suganya and Senthil Kumar 2018). Activated carbon produced by the carbonization of biomass (e.g. coconut shell wood waste, chubby grains, spent grains; Amerkhanova et al. 2017, Belc et al. 2019) is called biochar. As the production of biochar from by-products converts hard-to-remove waste into an adsorbent for pollutant treatment, its use for extract concentration is considered a doubly effective solution in the development of sustainable waste valorization technologies.

So far, polyphenols have been recovered from raw materials such as fruit, vegetables and cereals (Garcia-Salas et al. 2010, Skrajda-Brdak et al. 2019, Montenegro-Landívar et al. 2021) and such waste as olive pomace, residues of a winery, pomegranate skins, citrus waste, rice bran and soybean residues, other than stillage coming from cereal processing into alcohol (Tapia-Quirós et al. 2022b, Selvakumar et al. 2021, More and Arya 2019, Yadav et al. 2022, Pourali et al. 2010, Nkurunziza et al. 2019). Based on the literature presented above, it can be concluded that the recovery of phenolic compounds should be optimized for each type of waste material in terms of extraction conditions and types of solvents. To the best of author's knowledge, there are no reports of research on the use of THFA as a "green" solvent and biochar (both derived from a solid fraction of cereal processing waste) to recover and concentrate phenolic acids from the liquid fraction of distillery stillage. In addition, the literature on the effect of polyphenols on biogas production is scarce; the type and concentration of phenolic acids that adversely affect the bio-methanization should be specified to conclude about the rationality of polyphenol recovery from distillery stillage before its anaerobic treatment.

2. Work objectives and hypotheses

The objective of the study presented in the series of publications, which constitute the scientific achievement, was to determine the potential of distillery stillage as a source of bioactive polyphenols, the possibilities of their recovery, and the effect of phenolic acids on the methane potential of distillery stillage.

The scope of the studies involved:

1. Characteristics of distillery stillage and methods of its treatment with special attention to its valorization by recovering compounds with antioxidant activity, according to the biorefinery concept.
2. Determining the effect of extraction methods (CSLE, MAE, UAE), type and concentration of organic solvents, solid-to-solvent ratio and the extraction time on the content of polyphenols recovered from distillery stillage, the type and content of free and bound phenolic acids, extraction kinetics and the antioxidant activity of the extracts.
3. The use of SWE for the recovery of polyphenols from distillery stillage: determining the effect of the temperature, extraction time and solid-to-solvent ratio on the content of polyphenols, the type and content of free and bound phenolic acids, extraction kinetics and the antioxidant activity of the extracts, and the effect of extraction temperature on the composition of the solid residue remaining after extraction.
4. Investigating the possibility of the recovery of phenolic acids from the liquid fraction of distillery stillage *via* the synergistic use of THFA as a solvent and biochar as an adsorbent, which were produced from the solid fraction of cereal processing waste.
5. Investigation of the effect of the type and concentration of the individual phenolic acids and their mixtures on the methane potential of the distillery stillage, the methane percentage in the biogas, the methane production rate, and the abundance of methanogenic Archaea in the microbial communities of distillery stillage.

The formulated research scope allowed for the verification of the following research hypotheses:

Hypothesis 1: Methods and conditions of extraction and the type and concentration of the solvents affect the yield, composition and antioxidant activity of polyphenols recovered from distillery stillage.

Hypothesis 2: THFA and biochar produced from the solid fraction of cereal processing waste can be used for the recovery of phenolic acids from the liquid fraction of this waste.
Hypothesis 3: The methane potential of distillery stillage is affected by the type and concentration of phenolic acids.

3. Materials and methods

The studies included in the Ph.D. thesis were divided into three research series. Series I involved the extraction of polyphenols from the distillery stillage with commercial organic solvents (ethanol, methanol, acetone, and ethyl acetate) by using CSLE, UAE MAE and water as a solvent by using SWE. Series II involved the use of THFA and biochar produced from the solid fraction of cereal processing waste (including distillery stillage) to recover phenolic acids from the liquid fraction of the stillage. In series III, the effect of phenolic acids on the methane potential of distillery stillage was studied.

The distillery stillage used in each experimental series was a by-product of the full-scale production of concentrated crude ethyl alcohol from cereals (including wheat and rye) in a distillery located in Poland ("53°30′00″ N, 20°10′19″ E"). The characteristics of the stillage were the following: 47,000±5,300 mg COD/L, 4,345±5 mg N_{total}/L, 280±2 mg P_{total}/L, 789±3 mg VFA (volatile fatty acids)/L, 4.52 pH and the moisture of 93.6±2.8%.

In series I, freeze-dried distillery stillage (the moisture of 9.8±0.9%) was used. The conditions of extractions with CSLE, UAE, MAE, and SWE, determined on the basis of literature data and preliminary studies, are shown in Table 1.

Characterization of the obtained extracts included: total polyphenol content (TPC) (Singletion et al. 1999), total flavonoid content (TFC) (Quettier-Deleu et al. 2000), the types and content of total and free phenolic acids, and the antioxidant activity using three assays (DPPH (Moure et al. 2001), ABTS (Re et al. 1999), and FRAP (Benzie and Strain 1996)). The results were related to dry mass (DM). To determine the content of total phenolic acids, hydrolysis was carried out. The free and total phenolic acids were identified and quantified by High-Performance Liquid Chromatography (HPLC) (P2, P3, P4, P5). The performance of the procedure for identifying phenolic acids was assessed by linearity, accuracy, the limit of detection (LOD), and the limit of quantification (LOQ)

(P2). The biomass remaining after SWE was characterized in terms of its elemental composition (P5).

Table 1. Conditions of polyphenol extraction from distillery stillage using CSLE, UAE, MAE and SWE

Method of extraction	Type of solvent	Concentration of the solvent (%, v:v)	Solid-to-solvent ratio (w:v)	Extraction time (min)	Extraction temperature (°C)
CSLE	ethanol	60, 70, 80, 90, 100	1:30	15, 30, 45, 60, 90	room temperature
	methanol				
	ethyl acetate:ethanol:water	100:0:0, 90:0:10, 80:10:10, 70:20:10, 60:30:10	1:15, 1:30		
UAE	ethanol	60, 70, 80, 90, 100	1:30	3, 5, 10, 15, 20	room temperature
	methanol				
	acetone	40, 60, 80, 100	1:15, 1:30		
	ethyl acetate:ethanol:water	100:0:0, 90:0:10, 80:10:10, 70:20:10, 60:30:10			
MAE	ethanol	60, 70, 80, 90, 100	1:30	1, 3, 5, 8, 10	50
	methanol				
	acetone	40, 60, 80, 100	1:15, 1:30		
	ethyl acetate:ethanol:water	100:0:0, 90:0:10, 80:10:10, 70:20:10, 60:30:10			
SWE	water		1:5, 1:15, 1:30, 1:50	5, 15, 30, 60, 90	25, 80, 140, 200, 260

In series II, for the recovery of phenolic acids from the liquid fraction of distillery stillage, THFA and biochar produced from the solid fraction of distillery stillage and cereal bran were used. Firstly, the UAE of phenolic acids from distillery stillage with 80 and 100% THFA at 15 min was carried out. The phenolic acids from the extracts were adsorbed on different doses (17, 34, 85 and 170 g/L) of biochar and commercial powdered activated carbon. The desorption of phenolic acids from adsorbents was carried out by

water (40, 50, and 60°C). In the THFA extracts and water after desorption, phenolic acids and antioxidant activity (ABTS, DPPH and FRAP) were determined with the same methods as in series I (P7).

In series III, the effect of phenolic acids on the methane potential of distillery stillage was measured in an automatic methane potential batch-test system (AMPTS) for 21 days. The effects of individual phenolic acids (p-OH benzoic, vanillic, ferulic, sinapic, syringic, and *p*-coumaric) and their mixture on biomethanization were investigated. Individual phenolic acids and their mixtures were added to the feedstock for final concentrations of 0.5, 1.0, 1.5, 2.0, and 4.0 g/L. In the samples of stillage before and after fermentation (digestate), the contents of total solids (TS), volatile solids (VS), COD, ammonium nitrogen, VFAs, alkalinity, P_{total}, pH, total organic carbon (TOC), dissolved organic carbon (DOC) and TPC were measured. The biogas composition was determined with a gas chromatography (GC). To determine the effect of phenolic acids on methanogenic Archaea, the biomass was analyzed with fluorescence *in situ* hybridization (P6).

Calculation and statistical methods included:

1. The Spearman's rank correlation coefficient was used to quantify the relationships between the polyphenol content, phenolic acid content, antioxidant activities of the extracts and the extraction conditions (P2, P3, P4, P5, P7) and between the operating conditions, methane production and microbial abundance (P6).
2. To explain the degree of interaction between solvent concentration and yield of phenolic compounds by assessing the solubility of particular phenolic acids in the solvent, the Hansen Solubility Parameter (HSP) was employed (P3, P4, P7).
3. The second-order kinetics was used to describe the effect of extraction conditions on the extraction rate (P3, P4, P5) and the effect of phenolic acid type and content on the methane production rate (P6).
4. Principal Component Analysis (PCA) was used to determine the relationships between the TPC, TFC, phenolic acid content, antioxidant activity, and extraction conditions (P4, P5, P7) and between the operating conditions, methane production and microbial abundance (P6).
5. To optimize the extraction conditions, Response Surface Methodology (RSM) was used (P5, P7).

4. Description of experimental results and verification of research hypotheses

Hypothesis 1: Methods and conditions of extraction and the type and concentration of the solvents affect the yield, composition and antioxidant activity of polyphenols recovered from distillery stillage.

The potential of distillery stillage as a source of bioactive compounds was characterized in P1. Methods and conditions of extraction and the type and concentration of the solvent were evaluated based on yield and composition of polyphenols recovered from distillery stillage, rate of phenolic acid extraction and antioxidant activity of the obtained extracts (P2-P5).

By verifying hypothesis 1, it was shown that:

Distillery stillage, as a waste from cereal processing into alcohol, was a source of bioactive polyphenols. The content of polyphenols and phenolic acids in the extracts and their antioxidant activity depended on the extraction technique, type and concentration of the solvent and extraction conditions.

MAE and UAE can be considered "green" alternatives to CSLE because these methods provided more efficient recovery of polyphenols and higher antioxidant activity than CSLE at a significantly shorter time of extraction. For example, with ethanol, MAE and UAE allowed for 2.1- and 1.7-times more efficient polyphenol recovery, 15- and 9-times shorter extraction times and 2.1- and 1.5-times higher antioxidant activity, respectively, than CSLE. Depending on the solvent type, MAE or UAE can be indicated as the most efficient extraction technique. MAE was the most effective when methanol, ethanol and ethyl acetate were used. When using acetone as a solvent, the content of extracted polyphenols was about 1.2-times higher in UAE than in MAE; however, due to about a 3-times higher extraction rate (solid-to-solvent ratio of 1:15, 60% acetone), MAE had a better balance between extraction yield and energy consumption than UAE.

Each solvent allowed for the recovery of polyphenols from distillery stillage. However, the use of ethanol and water under subcritical conditions resulted in the highest content of polyphenols in the extracts. Independently of the extraction technique, ethanol was found to be the most effective solvent in recovering polyphenols. The efficient use of SWE for recovering allowed for the elimination of organic solvents from the extraction process and thus solvent residues from the extracts. This would make purification of the

extracts easier and is desirable for the use of recovered compounds in the pharmaceutical, cosmetics, and food industries.

Selecting the appropriate solvent mixture and thus modifying their polarity improved the extraction yield; the addition of water into all organic solvents increased the content of polyphenols in the extracts. The most effective solutions were: 80% (ethanol and methanol), 60% (acetone), 70% (ethyl acetate). This was confirmed by the results of the HSP analysis. An increase in the concentration of solvents reduced the efficiency of polyphenol recovery.

The most efficient times of extraction were 45 min (CSLE), 5 min (UAE) and 3 min (MAE) with ethanol and methanol; 10 min (UAE) and 5 min (MAE) with acetone; 60 min (CSLE), 15 min (UAE) and 8 min (MAE) with ethyl acetate; 30 min with water (SWE). Lengthening of the extraction times decreased the content of polyphenols in the extracts, which indicated their degradation.

Independently on the concentrations of polyphenols in the extracts, the antioxidant activity is the most valuable measure of the quality of the obtained extracts. For each solvent, the activity was strongly positively correlated with the content of total polyphenols and phenolic acids. The use of three different assays for determining the activity gave a comprehensive picture of the bioactive properties of the obtained extracts and allowed to conclude that polyphenols that were efficient at scavenging radicals predominated over those with a reducing power. The extracts that were characterized by the highest antioxidant activities resulted from the MAE with 80% ethanol, 80% methanol and 70% ethyl acetate. Despite high polyphenol content in the extracts after SWE, their antioxidant activity was about 4 times lower.

Independently of the extraction conditions, phenolic acids that were recovered were present mainly in the free form (up to 88–95% of the total), which allows to avoid hydrolysis of the stillage before the extractions. Of these acids, ferulic and *p*-coumaric acids (both belonging to hydroxycinnamic acids) predominated. Thus, it can be speculated that they had the greatest effect on the antioxidant activities of the extracts. Such a profile of phenolic acids opens various possibilities for the industrial applications of the obtained extracts.

Apart from the extraction time, the temperature had the greatest effect on the content and composition of polyphenols and the antioxidant activity of the extracts in SWE. The most effective SWE was at 140°C. The concentration of phenolic acids was 1.8–2.3-times higher in the extracts obtained at 140°C than at 260°C. SWE changed

chemically the distillery stillage; processing distillery stillage at 260°C increased its carbon content by 22% and decreased its oxygen content by 40%, which may indicate the conversion of stillage during SWE into a solid product termed hydrochar. As a result of stillage carbonization, its calculated heating value increased by 33%. Thus, the biomass remaining after extraction of polyphenols from distillery stillage with SWE can be considered a valuable product that can be used as a solid fuel, soil amendment or precursor of activated carbon. Because the most effective temperatures for polyphenol extraction and for obtaining the highest heating value of the biomass differed significantly, the SWE temperature should be selected depending on the desired output.

Hypothesis 2: THFA and biochar produced from the solid fraction of cereal processing waste can be used for the recovery of phenolic acids from the liquid fraction of this waste.

The studies presented in P7 report an innovative approach that combines the use of THFA and biochar produced from the solid fraction of cereal processing waste (including a solid fraction of distillery stillage) to recover phenolic acids from the liquid fraction of the stillage. THFA produced from furfural was used as a solvent in the UAE of phenolic acids from the distillery stillage. The extracted phenolic acids were recovered from the extracts using biochar generated by the carbonization of the solid fraction of the waste. The effect of THFA concentration on the extraction efficiency, the composition of phenolic acids and the antioxidant activity of the extracts, and the effect of the conditions of phenolic acid adsorption/desorption (biochar dose and desorption temperature) on the recovery of phenolic acids from the extracts and the resulting antioxidant activity were determined.

By verifying hypothesis 2, it was shown that:

Waste-derived THFA and biochar were suitable for the recovery of phenolic acids from distillery stillage and may replace commercial solvents and adsorbents in recovering phenolic acids from the stillage. THFA concentration, biochar dose and desorption temperature affected the recovery efficiency. The content of phenolic acids in the extracts was the highest with 80% THFA; adding water to the solvent increased the content of phenolic acids by 15%. The content of phenolic acids in the extract obtained with the use of THFA as a solvent was higher than those obtained with acetone, ethyl acetate and water, similar to that obtained with methanol and lower by about 7% than that obtained with ethanol. Similarly to the use of commercial organic solvents and water, mainly

hydroxycinnamic acids were extracted; ferulic acid contributed in almost 44% and *p*-coumaric acid in about 22% of the total phenolic acids. The antioxidant activity of THFA extracts was similar to the extracts obtained with methanol and ethanol, 15% lower than that obtained with ethyl acetate and much higher than those obtained with acetone and water. Phenolic acid recovery from the extracts in adsorption/desorption with biochar was 60–94%, depending on the biochar dose. The content of total phenolic acids in water after desorption decreased with a lowering of biochar dose. Phenolic acid recovery from the extracts was the highest when desorption was performed at 50°C; further increase in water temperature decreased the efficiency of desorption. In the desorbed fraction from biochar, *p*-coumaric and ferulic acids predominated (15–21% and 29–41% of the total phenolic acids, respectively), which had the greatest effect on the antioxidant activity. After adsorption/desorption, ~95% of the antioxidant activity of phenolic acids in the extracts was maintained. Similar results of adsorption/desorption of phenolic acids from the extracts were obtained with biochar and commercial powdered activated carbon. The study proved that synergistic processing of different streams of waste coming from cereal utilization for alcohol production may be considered a direction in waste-to-profit management of waste from the agro-food industry.

Hypothesis 3: The methane potential of distillery stillage is affected by the type and concentration of phenolic acids.

The studies presented in P6 assessed the effect of the type and concentration of individual phenolic acids (p-OH benzoic, vanillic, ferulic, sinapic, syringic, and *p*-coumaric) and their mixtures on the methane potential of distillery stillage, the methane production rate, and the abundance of methanogenic Archaea in the microbial communities. The relationship between the chemical structure of individual phenolic acids and their effect on biomethanization was described.

By verifying hypothesis 3, it was shown that:

Phenolic acids affected microbial growth and inhibited methanogenesis, thus decreasing the methane potential and the rate of methane production. The negative effect of phenolic acids on the anaerobic treatment of distillery stillage was strongly dependent on the type of acid (its chemical structure) and the concentration. At low concentrations (< 1.0 g/L), about 80% of phenolic acid content were utilized by microorganisms as a carbon source. After exceeding this concentration, phenolic acid degradation was stopped and then they started to inhibit anaerobic processes. With 1.5 g/L of phenolic

acids, methane production was 43–61% lower than in the control sample (distillery stillage without phenolic acids added); the methane production accounted for 34.0–49.6% of the maximum theoretical methane potential, depending on the type of acid. 2.0 g/L of individual vanillic, *p*-coumaric, syringic and ferulic acids inhibited methane production completely. Spiking distillery stillage with 4.0 g/L of all individual phenolic acids resulted in no methane production. The inhibition constant for methane production was 0.5–1.0 g/L of an individual phenolic acid or 1.5 g/L of the acid mixture. Vanillic, *p*-coumaric, syringic and ferulic acids limited methane production from distillery stillage the most. An increase in the concentration of phenolic acids from 0.5 to 4.0 g/L resulted in a decrease in the abundance of methanogenic Archaea by about 30 times; independently of operating conditions, methanogenic Archaea was predominated by acetoclastic methanogens. Due to biomass exposition to increasing amounts of phenolic acids, the abundance of hydrogenotrophic microorganisms decreased, which could have changed the conditions in the anaerobic biomass leading to the imbalance between VFA-producing and VFA-consuming microorganisms and the accumulation of VFAs in the digestate.

5. Summary

It was shown that distillery stillage was a source of polyphenols. Hence, the recovery of these bioactive compounds has been proposed as an option for valorizing the stillage. The yield and composition of polyphenols, extraction rate, and antioxidant activity as a measure of the extract quality depended on the type and concentration of the solvents and the extraction methods and conditions. Because of more efficient recovery of polyphenols, higher antioxidant activity and a significantly shorter time of extraction, MAE and UAE were found to be "green" alternatives to the conventional extraction. Although ethanol was the most effective solvent in extracting polyphenols, special attention should be paid to the use of water under subcritical conditions. This extraction technique resulted not only in a high content of polyphenols in the extracts, with simultaneous elimination of organic solvents from the extraction process, but also in transforming the waste biomass into a valuable product, hydrochar, that can find different applications. However, due to different conditions that are optimal for polyphenol extraction and hydrochar generation, the conditions of SWE should be selected depending on the desired output.

The proposed valorization concept consists of the multidirectional use of waste biomass from distilleries. The attempt of application of THFA and biochar derived from the solid fraction of cereal processing waste to recover phenolic acids from its liquid fraction indicated that different streams of cereal waste may be synergistically processed. These promising results can lay the foundation for development of waste-to-profit management of agro-food waste.

In the study, the recovered polyphenols have been characterized in terms of their form, composition and the type of the antioxidant activity, which is a base for further studies on their applications in various industries. In addition, by determining the maximum allowable levels of phenolic acids in anaerobic systems used for distillery stillage valorization, the results may be valuable for improving biogas production from wastes of different compositions and concentrations of bioactive compounds.

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6. Abbreviations and Symbols

ABTS – 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

BOD – biochemical oxygen demand

COD – chemical oxygen demand

CSLE – conventional solid-liquid extraction

DM – dry mass

DOC – dissolved organic carbon

DPPH – 2,2-diphenyl-1-picrylhydrazyl

FRAP – iron reducing antioxidants

GAE – gallic acid equivalent

GC – gas chromatography

HAT – hydrogen atom transfer

HPLC – High-Performance Liquid Chromatography

HSP – Hansen Solubility Parameter

LOD – limit of detection

LOQ – limit of quantification

MAE – microwave-assisted extraction

PCA – Principal Component Analysis

QUE – quercetin equivalent

RSM – Response Surface Methodology

SET – single electron transfer

SWE – subcritical water extraction

TE – Trolox equivalent

TFC – total flavonoid content

THFA – tetrahydrofurfuryl alcohol

TOC – total organic carbon

TPC – total polyphenol content

TS – total solids

UAE – ultrasound-assisted extraction

VFA – volatile fatty acids

VS – volatile solids

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Attachment 1.

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Distillery Stillage: Characteristics, Treatment, and Valorization

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Abstract
Distilleries are among the most polluting industries because ethanol fermentation results in the discharge of large quantities of high-strength liquid effluents with high concentrations of organic matter and nitrogen compounds, low pH, high temperature, dark brown color, and high salinity. The most common method of managing this wastewater (distillery stillage) is to use it for soil conditioning, but this requires thickening the wastewater and may cause soil pollution due to its high nitrogen content. Therefore, treatment of distillery stillage is preferable. This review discusses individual biological and physicochemical treatment methods and combined technologies. In addition, special attention is paid to valorization of distillery stillage, which is a valuable source of polysaccharides and volatile fatty acids (VFAs), as well as natural antioxidants, including polyphenols and other bioactive compounds of interest to the pharmaceutical, cosmetic, and food industries. New directions in improvement of valorization technologies are highlighted, including the search for new eutectic solvents for extracting these compounds. Such technologies are essential for sustainable development, which requires the use of management and valorization strategies for recovery of valuable compounds with minimal disposal of waste streams.

Keywords Distillery stillage · Valorization · Recovery of bioactive compounds · Polyphenols

Distillery Industry

Alcohol is considered a basic material for a number of industries, e.g., the chemical, pharmaceutical, cosmetics, beverage, food, and perfume industries, and the number of alcohol distilleries is increasing worldwide [1]. In 2005–2007, ethanol production increased by about 41% (from 44.3 to 62.6 billion liters) [2]. The largest producers of bioethanol in the world

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include developed countries (such as the USA, and in the European Union, Germany, and France) and developing countries (Argentina, China, India, Indonesia, and Malaysia) [3]. In recent years, ethanol production has increased because the European Union has implemented a renewable fuels program, which obliges Member States to use renewable fuels. The share of biofuels from non-food substrates in transport fuels will continue to increase and should reach 10.3% in 2020, 14.0% in 2025, and 19.7% in 2030 [4].

Currently, alcohol production is based on the use of non-waste agricultural substrates, such as cereals, potato starch, molasses from sugar beet, beetroot, and fruits [5]. Ethanol fermentation can be performed with any naturally occurring sugar, starch, or cellulosic material combined with the appropriate pretreatment. Whereas sugary raw materials that contain carbohydrates can be directly assimilated by yeast, starchy raw materials require acid or enzymatic hydrolysis to glucose or fructose before ethanol fermentation [6]. On a global scale, about 47% of ethanol is produced from sugary raw materials [7]. In Europe, Poland is one of the biggest generators of sugar [8], which is produced from sugar beet with a yield of 8.58 t/ha; 2065.3 thousands of tons was produced in 2019/2020 [9]. As a by-product of sugar production, molasses is formed. Molasses contains 30% water and can be stored in tanks for a couple of months [10]. In 2007, 539 million liters of bioethanol from sugar beets and beet molasses was produced in France [5]. A global leader in the production of bioethanol is Brazil. In 2007, 21.3 billion liters of alcohol was produced, using molasses and sugar cane juice [11]. Most distilleries partner with sugar mills and make use of molasses from cane sugar manufacturing as the starting material for alcohol production. Although various biomass materials can be used to prepare ethanol, their usefulness as feedstocks depends on their cost, availability, carbohydrate content, and the ease by which they can be converted into alcohol [12].

Four stages of alcohol production in distilleries embrace feed preparation, fermentation, distillation, and packaging [13]. The feed, or fermentation broth, for ethanol production contains raw materials and nitrogen and phosphate supplements. Fermentation is performed by yeast, for example *Saccharomyces cerevisiae* [14], which convert the carbohydrates into alcohol and then settle in a sludge at the end of the process. The mass after fermentation contains 8–10% ethanol. In a distillation column, the alcohol is separated out as the top product, and the bottom product is a brownish liquid known as distillery stillage [15]. In general, distillery stillage (spent wash) and water for the system cooling are the main contributors in total volume of wastewater produced in particular steps of alcohol production (Table 1).

Distillery stillage may pose a serious environmental concern since it pollutes the water sources in several ways. First, the dark-colored stillage can block out sunlight, inhibiting photosynthesis and reducing the oxygenation of the water, which is detrimental to aquatic life

Table 1 Wastewater produced in distilleries

Operations in distilleries	Liter wastewater/liter alcohol	References
Spent wash	11.9	[1]
Fermenter cleaning	1.6	[16]
Fermenter cooling	7.2	[13]
Condenser cooling	7.9	[16]
Floor wash	1.1	[15]
Bottling plant	1.3	[15]
Others	0.8	[16]

[17]. Second, a high pollution load causes eutrophication of water bodies. For these reasons, untreated distillery stillage causes depletion of dissolved oxygen in water bodies and harms aquatic flora and fauna [1]. Currently, in some countries, by-products from the distillery industry are used for direct soil fertilization (spreading on the fields and plowing) due to their high content of nitrogen, phosphorus, and organics. As an example, vinasse generated from sugar cane juice fermentation is mainly used as a fertilizer in Brazil [13, 18]. Deep well disposal was another option for disposal of by-products, but due to limited storage capacity and to pollution of underground water, this was not considered a good option.

Due to the production of a large amount of wastewater (12 times as much by volume as the alcohol produced) of very high organic loads [19, 20], distilleries are highly water intensive units. Environmental and economic costs can be optimized through rational management of this effluent. Therefore, according to the principles of a bioeconomy, distillery stillage should be transformed into valuable products [21], like heat, power, or feed and other bioproducts, such as compost [22–24]. For all these reasons, it is not only important to purify distillery wastewater but also to use it as a feedstock for the production of value-added products.

Characteristics of Distillery Stillage

The pollution of distillery stillage depends on the quality of the substrates and the unit operations used for alcohol production, which means that the characteristics of stillage can differ between distilleries [15, 25]. For every liter of alcohol produced, molasses-based distilleries usually generate about 8–15 L of stillage characterized by high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) [26, 27]. The COD and BOD values of this stillage are due to the presence of a number of organic compounds, such as polysaccharides, proteins, polyphenols, waxes, and melanoidin [28]. Distillery stillage contains about 2% of natural products of sugar and amino acid condensation known as melanoidins [29], which contain a lot of dark brown pigment. These substances lead to environmental pollution [30] and have antioxidant activity, which makes them toxic to microorganisms that are present in wastewater treatment processes [31]. The conventional treatment methods are not enough to eliminate melanoidins [32]. Therefore, treated distillery wastewater still contains almost the same dark brown color as before treatment because of the non-biodegradability of the colored compounds [33]. This is one of the reasons why distillery wastewater is difficult to treat. Distillery stillage has high levels of nutrients, such as nitrogen, phosphorus, potassium, and also of total solids, total dissolved solids, total suspended solids, sulfate, and iron. Table 2 presents the physical and chemical properties of wastewater from the distillery industry [1, 34, 35].

The discharge of wastewater produced by distilleries has become increasingly restricted as the requirements of environmental regulations have become more stringent and as understanding grows regarding the negative effects of seasonal discharges of water containing high nutrient and organic loadings into water courses [35]. Numerous environmental directives and regulations have been issued, and laws have been passed to define quality standards for water. To abide by stringent government policies on pollution control, industries have now been forced to look for more effective treatment technologies that are not only environmentally friendly but also cost-effective [36]. Hence, distilleries have faced problems in the management of the wastewater they produce and it has become a challenge to meet the requirements for this wastewater. Treatment of distillery wastewater is difficult and expensive. Many

Table 2 Physical and chemical characteristics of distillery stillage

Characteristic	Units	Value
pH	–	4.0–4.5
Temperature	°C	71–81
Color	–	Dark brown
Total solids	mg/L	59,000–82,000
Volatile solids	mg/L	38,000–66,000
Total suspended solids	mg/L	2400–5000
COD	mg/L	100,000–150,000
BOD	mg/L	35,000–50,000
VFA	mg/L	2300–2400
Nitrogen	mg/L	1660–4200
Phosphorus	mg/L	225–308
Potassium	mg/L	9600–15,475
Iron	mg/L	1550–1800
Sulfates	mg/L	2100–2300
Calcium	mg/L	2300–2500
Magnesium	mg/L	220–250

researchers have been investigating technologies for the treatment of distillery wastewater; however, none of these have been found to be effective and economically viable methods of complying with regulations [1].

Distillery stillage can be considered a pollutant, but it has also been used in agriculture. For this application, distillery wastewater is potentially valuable, but it also poses problems and has negative effects on the environment. At high doses (> 250 m^3/ha), the use of distillery stillage is harmful to plant growth and soil properties, but its application at lower doses (125 m^3/ha) significantly improves the sprout, growth, and yield of dry land plants, due to its content of nutrients (P, N, K, and Ca). Moreover, the combined application of distillery stillage and natural organic compounds (cattle manure, green leaf manure, and bio-compost) is suitable under dry land conditions [23].

Treatment of Distillery Stillage

From environmental point of view, elimination of organics from by-products from distillery industry is becoming increasingly important in terms of reduction of soil and water pollution. The treatment of distillery effluents is a challenging issue due to the presence of difficult-to-degrade compounds such as caramel, sugar degradation products, anthocyanins, tannins, and different xenobiotic compounds [25] and their toxic effect on humans and the environment. Such compounds are not effectively removed by conventional biological treatment processes. Hence, several physico-chemical, biological, and integrated treatment processes for effective degradation of organic matter have been explored to address the demands of environmental regulations [18]. The technological methods used for the treatment of distillery stillage are biological (aerobic and anaerobic), physico-chemical (coagulation/flocculation, electrocoagulation, adsorption, advanced oxidation, and membrane processes), and thermal (evaporation/combustion). These methods have been practiced and examined during recent years. The choice of treatment method depends on its efficiency and cost, the type of land where the distillery stillage will be used after treatment, regulatory constraints, and public acceptance of the treatment.

For the complete treatment of distillery stillage, individual methods and technologies should not be used, but instead a comprehensive treatment that involves several sequential technologies should be employed. Such an approach provides multiple benefits in terms of environmental protection, energy conservation, and production of high value compounds. Therefore, in the descriptions below, combined technologies are given in addition to individual processes.

Biological Treatment

Biological treatment of distillery stillage depends on the natural growth and selection of microorganisms. During this process, the microorganisms utilize pollutants for growth and convert the organic substrates into simpler substances in the presence or absence of oxygen. Both methods (aerobic and anaerobic) can be used separately for the treatment of distillery stillage, but because this kind of wastewater has high concentrations of organic pollutants, in most cases, a combination of both is used.

Anaerobic Treatment

Anaerobic digestion is a natural process in which anaerobic microorganisms utilize organic matter and transform it to biogas, which makes this treatment more attractive than aerobic treatment. The process is conducted by a comprehensive ecosystem in which physiologically different groups of microorganisms function and interact with each other. A number of microorganisms are involved in anaerobic digestion, including acetic acid-forming bacteria (acetogens) and methane-forming archaea (methanogens). The anaerobic digestion can treat high-strength distillery stillage more effectively than aerobic treatment because it degrades concentrated wastewater, produces a small amount of sludge, requires less energy, and produces economically valuable bioenergy (biogas) [15]. The by-products from the alcohol industry with a minimum COD concentration of 2800 mg/L can be used in fermenters for biogas production [1]. Furthermore, the anaerobic process is vulnerable to organic load and low pH, and the anaerobic microorganisms grow slowly, which leads to longer hydraulic retention time necessary.

Anaerobic processes in the treatment of distillery stillage have been used in various reactor technologies. UASB reactors have been reported to be particularly successful in the treatment of highly polluted effluents. This technology is attractive because the reactors do not require mechanical mixing and enable recycling of biomass sludge. According to Wolmarans and De Villiers [37], UASB technology not only removed over 90% of COD when treating distillery stillage that contained about 30,000 mg/L of COD but also produced biogas with the productivity of 0.43 m^3/kg. The process should be carefully controlled by first operating at a low loading rate (4–8 kg COD/(m^3 day)) to ensure successful startup, and then, when removal efficiency is over 90%, increasing the loading rate up to even 30 kg COD/(m^3 day) is possible. On the basis of these results, we can deduce that the distillery stillage is a potential source for the production of biogas, and that anaerobic digestion is a reliable solution for the treatment of this type of effluents.

Because anaerobic bacteria are capable of transforming distillery stillage, which is rich in organic compounds, into biogas, anaerobic digestion is highly advantageous for the treatment of such effluent. The process demands less energy input and also very low nutrient load. Generation of energy can reduce operational costs to a large degree as compared with energy-intensive aerobic processes [38].

Aerobic Treatment

Although the key disadvantage of the aerobic processes for the treatment of distillery stillage is high energy consumption, these processes are widely used because of their high efficiency and ease of use. They are applied both as a pretreatment and as a final treatment [39].

A large number of microorganisms (bacteria, cyanobacteria, yeast, fungi, etc.) can be used for treatment of distillery stillage in aerobic conditions. Filamentous fungi can be considered important phenolic-degrading organisms, as they frequently grow on wood, utilizing lignin as a carbon source [40, 41]. One of the first publications dealing with the distillery stillage treatment by aerobic methods comes from 1965 [42]. Distillery stillage was treated in a stirred reactor, achieving COD elimination of 60.7%. Other authors used a bioreactor with a fixed bed and recirculation of treated stillage and obtained much higher removal of COD of 98% [17]. The efficiency of treatment depended on the following factors: temperature, pH, COD, and nutrients (ammonia nitrogen and phosphate phosphorus) [7, 19]. For the treatment of distillery stillage coming from maize and rye, the variety of species of *Bacillus* bacteria (thermophilic and mesophilic) was used. In the case of stillage from maize, the removal of COD was 82.6%, but from rye residues, COD elimination was 84.6% [43]. Reports are also available on the aerobic treatment of distillery stillage from wheat grain [19]. The system yielded 64% COD removal at 45 °C and about 90% at 55 °C. Jackson et al. [44] used an aerated bioreactor to treat distillery stillage with a HRT of 14 days. COD was removed from 2255 to 150 mg/L.

Currently, study is focused on the combination of anaerobic and aerobic techniques for effective removal of pollutants from distillery stillage. Kapdan et al. [45] obtained 95% COD and 85% color removal efficiency in anaerobic-aerobic treatment of by-products from the distillery industry. On the other hand, Jimenez et al. [38] investigated the combined aerobic-anaerobic treatment of distillery stillage. The reason for such an order of processes was that phenolic compounds are considered toxic to methanogenic microorganisms and the high salinity can cause osmotic pressure problems to the methanogens. Therefore, in the first step, most of the phenolic compounds, color, and part of COD were removed under aerobic conditions. In the second step, anaerobic treatment eliminated the remaining organic content. Authors achieved COD removal at the level of 96.5%. The time necessary for decomposition of a given organic load, during using combined aerobic-anaerobic treatment, is lower than that necessary for a single treatment. To conclude, combined aerobic and anaerobic treatments lead to more efficient removal of color and COD from distillery stillage than individual conventional biological treatments.

Physico-chemical Treatment

Physico-chemical methods of distillery stillage treatment combine physical and chemical processes, in which the first process leads to the removal of suspended materials and the second to the elimination of soluble COD. Such processes involve coagulation/flocculation, electrocoagulation, adsorption, advanced oxidation, and membrane treatment [46].

Coagulation/Flocculation

Coagulation is a process of agglomeration of suspended particles present in wastewater by inorganic coagulants, such as sulfates and chlorides of iron, aluminum, or copper, or bioflocculants [47]. Prajapati et al. [46] determined the efficiency of removal of COD and

color from distillery stillage from a rice grain-based distillery with a COD concentration of 13,600 mg/L using $CuSO_4 \cdot 5H_2O$ at optimum pH of 6. It was shown that this coagulant ensured 91% of COD removal and 85% of color removal. COD and color removal increased as the dosages of coagulant were increased from 20 to 60 mM, but above this limit, increasing the dosage did not increase the percentage of COD removed. The treatment of distillery stillage by coagulation/flocculation led to COD removal of 55, 60, and 72% and color removal of 83, 86, and 92%, respectively, with the use of 60 mM aluminum chloride, 60 mM iron chloride and 30 mL/L of poly-aluminum chloride [48].

Chemical coagulation is an efficient pretreatment process when used before biological or membrane treatment processes or used as a final polishing treatment to eliminate non-biodegradable organic matter from by-products from distillery industry [49]. The disadvantages of coagulation are the high doses of required reagents, the unsatisfactory coagulant recovery rates, and the generation of sludge.

Electrocoagulation

Electrocoagulation is a complex, effective alternative to chemical coagulation process for the treatment of COD-rich wastewater. Electrocoagulation is based on the electrolytic dissolution of metals like iron or aluminum and *in situ* production of insoluble suspension which can remove the pollutants from the wastewater by coagulation. In surface complexation mode, the pollutant acts as a ligand to chemically bind hydrous iron or aluminum. In electrocoagulation, direct current is used [50].

Electrochemical treatment of rice grain–based distillery effluent using an iron electrode has been studied by Prajapati and Chaudhari [51] who investigated the effect of pH (3.5–9.5) on COD and color elimination. COD and color removal efficiencies were 83 and 69%, respectively, and the efficiency of organics removal was strongly dependent on the initial pH. Dayaca et al. [52] used electrocoagulation with an aluminum electrode for the removal of COD from distillery stillage, aiming at determination of the optimal current density, distance between electrodes, and pH. The treatment procedure was conducted using a constant electrolysis time of one hour. It was reported that a treatment with current density of 0.2 A/cm^2, distance between electrodes of 1 cm, and pH of 6 gave the highest COD removal of 79.09%.

This method has severe limitations due to the consumption of large amounts of electricity [53]. Other drawbacks include the production of chemical sludge, which requires additional measures for effective handling, storage, and utilization or disposal [54].

Adsorption

Adsorption on activated carbon (AC) is employed for the elimination of color and specific organic pollutants. The efficiency of adsorption technology depends on adsorbent pore volumes, which affect the reactions between the adsorbate and adsorbent, the nature of the adsorbent, and the type of activating agent and activation conditions [55]. AC is regarded an effective adsorbent due to its properties: increased surface area, microporous structure, and high degree of reactivity [13].

Bharagava and Chandra [3] reported decolorization of distillery wastewater that was achieved by using packed bed anaerobic reactor with AC of a surface area of 1400 m^2/g. Almost complete decolorization was obtained with 70% of the diluted sample. Lalov et al. [56] investigated the treatment of distillery stillage using chitosan as an adsorbent. During 30-min

process at an optimum chitosan dosage of 10 g/L, the removal of color and COD at the levels of 98 and 99%, respectively, was observed. Mall and Kumar [57] compared the color removal using AC and bagasse flyash. They noticed about 58% of color reduction with 30 g/L of bagasse flyash and 80.7% with 20 g/L of AC. In addition, the bagasse flyash has high carbon content and the adsorbed organic material further increases its heating value. Therefore, the spent adsorbent can be used for manufacturing fire briquettes.

The main disadvantage of adsorption for the treatment of distillery wastewater is high operational costs [36] and the production of high volume of solid waste [30].

Advanced Oxidation

Fenton process, oxidation, ozonation, and wet oxidation are advanced oxidation processes that can be used to treat distillery wastewater. Fenton process is based on generation of hydroxyl radicals (•OH) with extremely high oxidation potential, through catalysis of H_2O_2 by Fe^{2+} ion under acidic conditions. The electrochemically active organic compounds react with oxygen, which consequently leads to mineralization of color components and bioresistant fractions present in distillery stillage [51].

Solar photo-Fenton oxidation process showed a significant removal of COD, dissolved organic carbon (DOC), and color by 70.0 ± 3.3, 53.0 ± 3.7, and 75.0 ± 2.2%, respectively [58]. In an electro-Fenton process using iron electrodes for treatment of distillery wastewater, COD and total organic carbon (TOC) removal was at the level of 92.6 and 88.7%, respectively [59].

The highest efficiency of the treatment of distillery stillage with the use of advanced oxidation can be obtained with the combination of these processes with other methods. Ozone oxidation after aerobic treatment of distillery stillage eliminates most of the degradable organic compounds. The un-decomposed phenolic compounds and organic matter remaining after the aerobic treatment can be completely oxidized by ozone [60].

Ramana et al. [61] studied the effects of various operating parameters such as ozone flow rate (5 to 15 L/min), initial pH (2 to 10), current density (0.10 to 0.50 A/dm^2), and H_2O_2 concentration (50 to 500 mg/L) on the removal of COD and color. The results showed that 100% of color and COD removal could be achieved by ozone-electrocoagulation process with an energy consumption of 5.7 kWh/m^3 during four hours of reaction time. Hadavifar et al. [62] carried out the treatment of distillery wastewater in a hybrid reactor that combined ozone oxidation and adsorption by granular AC. This resulted in 74.23% of COD removal and 68% of color removal at pH of 2. The wastewater treatment by wet-air oxidation process can serve as a pre-treatment step to enhance the biodegradability and facilitate biogas generation in subsequent biological treatment processes [63]. However, these methods are highly energy intensive and hence expensive [61].

Membrane Treatment

Membrane processes are considered the most reliable technologies for the treatment of wastewater originating from different industries. These are physical processes that separate the feed stream into permeate and retentate, in which particles are separated on the basis of their molecular size and shape, with the use of specially designed semi-permeable membranes. Although there are a number of different methods of membrane treatment, the most suitable for the treatment of distillery stillage is pressure-driven membrane filtration. The application of microfiltration (MF) and ultrafiltration (UF) for the treatment of distillery

wastewater to produce water suitable for reuse in the bioethanol industry has been studied by Vasić et al. [64]. The efficiencies of the MF membrane for the removal of COD, dry matter, total nitrogen, and suspended solids were 80.4, 78.0, 80.4, and 100.0%, respectively. To improve the quality of the effluent, the MF permeate was further filtered through an UF membrane. The efficiencies of removal of the first three pollutants were increased to 90.0, 99.2, and 99.9%, respectively, and the composition of the final permeate indicated that it could be reused in the bio-ethanol production process. Sedimentation and UF were used to treat the winery effluent; from 7,937 mg C/L in the raw effluent, a reduction by 56.6% was obtained in the permeate [65].

For the treatment of distillery effluents, the combination of membrane techniques and biological treatment was used. For example, Satyawali and Balakrishnan [13] operated a membrane bioreactor (MBR) in a continuous mode using submerged 30-μm nylon mesh filters at the organic loading rates from 3.00 to 5.71 kg/(m^3 day). Up to 41% COD removal and up to 87% suspended solid retention were obtained. Because of high mixed liquor suspended solids of 10–12 g/L and long solids retention time, the low molecular weight compounds were degraded, whereas the high molecular weight compounds comprising the color imparting melanoidins remained unaffected. Similarly, high molecular weight compounds were not degraded when MBR operation was supported by powdered activated carbon (PAC) when treating sugarcane molasses based distillery wastewater [13]. However, organics removal was higher with PAC addition and the operation at higher loading rates (from 4.2 to 6.9 kg/(m^3 day)) was possible. Moreover, PAC addition increased the critical flux by 23%, which prolonged the period between membrane cleaning.

Thermal Treatment

The thermal treatment of highly polluted wastewater from distilleries is considered an economical and effective supplement to the anaerobic digestion and oxidation processes. Heating the stillage at high temperatures (160–250 °C) results in large amounts of organic substrate in the form of solid precipitates. This charred solid stillage has a high heating value (17–24 MJ/kg) and could be easily separated by filtration and then dried [66].

Evaporation/Combustion

Currently, numerous full-scale plants that use thermal treatment are operated. Combustion of distillery stillage is gaining interest. The treatment relies on the utilization of stillage as a fuel source. Direct combustion of distillery stillage is more effective if the stillage is pre-dried (to less than 55% moisture content). However, the technology is currently expensive, particularly when applied in small to medium size industries. The most prevalent challenge is the high energy requirement for the pre-drying stage and the treatment of toxic gas emissions [67].

The distillery stillage can be dried using hot air (180 °C), which leads to generation of a dry powder (calorific value of around 3200 kcal/kg) [68]. The powder is typically mixed with agricultural waste (20% volume) and burnt in a boiler. Currently, the use of combustion of distillery stillage in fluidized bed has overcome the restrictions caused by the stickiness of distillery stillage and its high sulfate concentration [13]. Combustion is also an effective method of on-site distillery stillage disposal as it is accompanied with the production of potassium-rich ash [69] that can be used for land application.

Table 3 presents the overview of single and combined methods of treatment of distillery residue with the operational conditions and efficiencies of organic compounds and color removal.

Emerging Treatment Technologies

In general, for the processing complex wastewater such as distillery stillage, the integrated application of many techniques is very practical and promising [85]; combining several processes, including thermal integration or advanced oxidation processes, followed by anaerobic digestion as well as adsorption, is still being investigated. In the development of modern technologies of distillery residue treatment, according to the rules of sustainable development, effectiveness and economic points still remains a challenge. Therefore, apart from integrated processes, emerging treatment approaches include improvements of membrane treatment and the use of microalgae.

Despite very high efficiency of organic compounds and color removal, the use of membrane filtration is restricted by membrane cost and high energy consumption for operating the pumps. The greatest effort is put into searching both for new membrane materials that will decrease membrane fouling, thus giving optimal permeate flux and maximal pollutant rejection, and for energy sources that will minimize the cost. For example, Lapišová et al. [86] investigated the use of combinations of ceramic membranes (pore diameter from 0.2 μm to 300 kDa), which are less prone to fouling. Other authors investigated the pretreatment of stillage with the use of natural coagulants to decrease the fouling of MF membrane [87]. In addition, to optimize the operating cost, studies are conducted to increase the efficiency of stillage treatment so that the permeate can be reused further. For example, distillery wastewater may be purified by the combination of UF and reverse osmosis (RO) to obtain high-quality effluent [88]. In addition, to optimize the cost, retentate produced in membrane treatment can be used as an addition to fertilizers or for biogas production [89]. To meet energy demand in the membrane installation, Ryan et al. [90] suggested using power from the anaerobic digester. Therefore, the combination of biological treatment with the membrane filtration (such as MBRs), which favors nutrient and energy recover, seems to be an effective perspective for the development of distillery residue treatment. The promising method of decreasing energy cost may be using solar energy, which is considered the most abundant renewable energy source. However, this solution has only been tested in municipal wastewater treatment plants [91].

Apart from physico-chemical processes of stillage treatment, the use of microalgae seems to be a method that will be developed in the future [92]. This is because microalgae can use waste as a source of nutrients and their by-products can be processed into alcohol, aquaculture feed, poultry feed, and organic fertilizers, thus reducing the total cost of treatment [93, 94]. However, combination of this treatment with other purification methods should be tested to increase the removal of color and odor [95]. Another challenge in the effective application of microalgae in the treatment of distillery residue is the selection of appropriate species of microalgae that effectively remove organic substances [96].

Recovery of Valuable Products from Distillery Stillage

To valorize distillery stillage, valuable and/or bioactive compounds can be extracted from it. Depending on the raw material used for fermentation, the composition of the stillage varies, as

Table 3 Overview of operational parameters and results of the treatment of distillery residue (OLR—organic loading rate, HRT—hydraulic retention time)

Treatment conditions	Treatment process	OLR (kg COD/(m^3 day))	HRT (day)	COD removal (%)	BOD removal (%)	Color removal (%)	References
Downflow fixed film reactor	Anaerobic	14.2–20.4	2.5–3.3	60–73	85–97	–	[70]
Upflow anaerobic sludge blanket reactor (25 °C)	Anaerobic	28	–	39–67	80	47	[71]
Upflow anaerobic sludge blanket reactor (30 °C)	Anaerobic	9–11	11–12	70	–	–	[72]
Downflow fluidized bed reactor with ground perlite (25 °C)	Anaerobic	4.5	3.3–1.3	85	–	55	[73]
Granular bed anaerobic baffled reactor (25 °C)	Anaerobic	4.75	4	90–96	80–92	–	[74]
Stirred reactor (35°°C)	Anaerobic	2–4	10.6–53.5	68.6–93.7	–	41	[38]
	Anaerobic after pretreatment with *P. decumbens*	2–4	3.1–15.4	63.5–82.6	–	41	
Stirred-tank reactor (38 °C)	Aerobic	104,000 mg/L (influent COD)	–	85.5	98.6	–	[75]
Reactor with shaking (22 °C)	Aerobic	2–4	5	50.7–52.7	–	41	[38]
Treatment with *P. sajor-caju* (30 °C)	Aerobic	42,000 mg/L (influent COD)	15	82.8	75.3	99.2	[76]
Reactor with shaking (30 °C)	Anaerobic and aerobic	62,000 mg/L (influent COD)	60	88	77.4	80	[77]
Two-stage bioreactor (stage I—alginate immobilized *P. putida*; stage II—batch culture of *Aeromonas* sp. (25 °C)	Anaerobic and aerobic	5	–	66	–	60	[78]
Stirred reactor (23–25 °C) (aluminum coagulant)	Anaerobic and coagulation/flocculation	38,000 mg/L (influent COD)	15	85	–	96	[79]
Upflow anaerobic suspended blanket reactor (iron coagulant)	Anaerobic and coagulation/flocculation	7,800 mg/L (influent COD)	–	87	92	99	[25]
Reactor with flowing oxygen and ozone (30 °C)	Advanced oxidation	59,000–62,000 mg/L (influent COD)	–	79	–	~ 100	[80]
Reactor with ozone-assisted electrocoagulation (30 °C)	Ozonation and electrocoagulation	80,000–90,000 mg/L (influent COD)	–	83 (4 h)	95 (4 h)	~ 100 (2 h)	[81]
Stirred reactor (iron coagulant; potential difference 5 V)	Coagulation/flocculation and electrochemical oxidation	93,650 mg/L (influent COD)	–	95	–	~ 100	[82]
Bioreactor with PAC (21–26 °C)	Adsorption	4.2–6.9	7	68	–	53	[83]
Membrane bioreactor (55 °C)	Membrane treatment		–	94.7	–	98	[84]

Table 3 (continued)

Treatment conditions	Treatment process	OLR (kg COD/(m^3 day))	HRT (day)	COD removal (%)	BOD removal (%)	Color removal (%)	References
Nanofiltration and reverse osmosis (55.2 bar; 25 °C)	Membrane treatment	40,000 mg/L (influent COD) 100,000 mg/L (influent COD)	–	~ 100	94	–	[27]

do the substances that can be recovered from them, i.e., polyphenolic compounds, polysaccharides, or volatile fatty acids (VFAs). In the fermented liquor, the content of bioactive substances is influenced by genetic factors, environmental factors, atmospheric conditions, exposure of substrates to stress, and the technological processes used [97]. Therefore, it is important to choose the optimal conditions for the recovery process. The most common techniques of substrate recovery are liquid-liquid extractions and solid-phase extractions [98]. The organic solvents using for recovery of substances are acetone, ethyl acetate, methanol, ethanol, and propanol or their mixtures. From an environmental standpoint, recovery techniques that meet the requirements of green chemistry are increasingly being sought. Therefore, the new alternative green methods and solvents are investigated for the recovery of valuable compounds. One of the options for green technologies could be supercritical fluids where mainly CO_2 [99] or water [97] are used; however, there are many drawbacks such as restricted range of molecule solubility, high cost of equipment, and production of pure water. Thus, this technology does not offer prospects for the future. Biomass-derived solvents, such as ethyl lactate, glycerol, or 2-methyl tetrahydrofuran, could be used as the next alternative. These solvents are renewable, non-toxic, and not very expensive, but their range of application and solvation properties are limited [100]. Considering the aforementioned statements, finding and developing green solvents, which will increase the efficiency of extraction processes, is a challenge. In conventional solvent extraction, mostly bioethanol is used, which is a biosolvent produced through alcoholic fermentation of sugar or starch-containing food wastes [101]. Extraction that is friendly for the environment should be characterized by reduced energy demands and the use of nontoxic solvents. Moreover, technologies should be characterized by highly effective recovery of substances, which would appear as a promising path towards sustainable production.

Recovery of Polyphenolic Compounds

Phenolic compounds are commonly found in plants. They have an important role in their growth, development, and reproduction and protect the plants against pathogens and predators. The polyphenol compounds are the most abundant antioxidants and include flavonoids (e.g., flavanols, condensed tannins, anthocyanins, or proanthocyanidins) and nonflavonoids (e.g., phenolic acids, lignins, stilbenes, ellagitannins and gallotannins) [102]. Moreover, polyphenol compounds have anti-inflammatory, analgesic, anticarcinogenic, and antimicrobial (antifungal and antiviral) properties [103]. At high concentrations, however, they are toxic [20]. The toxic effects depend on the concentration of polyphenols and the species of animal. In the case of fish, lethal effects are achieved at relatively low concentrations (5–15 mg/L). For different species, the average LD50 value is 300–600 mg/kg body weight [104]. However, due to the potential health benefits of these compounds, interest in using them in food, cosmetics, and medicine is increasing. Therefore, it could be important to recover phenolic compounds from distillery stillage and control the amount of these compounds that are released into the environment.

The most common method for the recovery of polyphenols is extraction. The main organic solvents for recovery of polyphenols are acetone and ethyl acetate or alkyl alcohols, e.g., methanol, ethanol, and propanol or their mixtures [105]. The final solvent composition is defined by the nature of the polyphenols and the type of sample used. Organic solvents have excellent dissolution abilities. However, they have many disadvantages, such as accumulation in the atmosphere (low boiling temperature), flammability, toxicity, non-biodegradability, and cost [106].

Librán et al. [107] determined the most effective operational conditions (treatment time and concentration of ethanol) of extraction of phenolic compounds from grape marcs. The phenolic compound content and antioxidant properties of the extracts were determined. The highest recovery of phenolic compounds (3.12 mg of gallic acid equivalent/g of grape marcs) was reported during 2 h of extraction in 75% EtOH liquid mixture at pH 2. Longer and shorter extraction times gave lower yield of the process. The best extraction yields were obtained with 75% ethanol solutions because a higher concentration of ethanol led to a decrease in the antioxidant and organoleptic properties of the phenolic compounds.

To increase the efficiency of polyphenol recovery, chemical substances were used to support ethanol extraction. Ross et al. [108] conducted alcoholic extraction of grain using ethanol:water extraction (80:20) at 4 °C for 5 h. They compared the efficiency of the extraction process with three methods of hydrolysis. In the first, alkaline hydrolysis was carried out with 10 M NaOH and then acid hydrolysis was carried out with 12 M HCl. In the second, alkaline hydrolysis was performed with 2 M NaOH, and in the third, acidic hydrolysis was conducted with 12 M HCl. Each of these hydrolysis methods had two variants. In the first variant, 2% ascorbic acid and 13.4 mM EDTA were added. In the second variant, these solutions were not added. The highest yield of phenolic compounds (3.46 mg of gallic acid equivalent/g of grain) was obtained using alkaline hydrolysis with the addition of 2% ascorbic acid and EDTA. These solutions protected the phenolic compounds from decomposition.

In addition, physical processes for increasing the extraction of polyphenols from distillery stillage have been tested. Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are gaining increasing acceptance owing to their benign character and the high effectiveness of recovery of various substances. The rapid penetration of microwaves into the solid leads to the generation of heat inside the solid and a rise in temperature. The heating is uniform, which accelerates extraction. UAE offers several advantages such as low cost, versatility, and ease of deployment [109] and may provide similar yields but with a significantly shorter resident time and lower temperature. Brahim et al. [110] investigated MAE of polyphenols from white grape pomace and red marc. They compared the effects of the following reaction conditions: temperature (60–120 °C), residence time (5–20 min), and the concentration of sodium carbonate (0–2.5%) that was used for decreasing degradation of anthocyanidins. With red marc, recovery was most efficient at 100 °C for 8 min without sodium carbonate. With white grape pomace, it was most efficient at 100 °C for 8 min with 2.5% sodium carbonate. The carbonate concentration did not affect phenolic compound extraction to a large extent. Drosou et al. [111] carried out the extraction of red grape pomace. Three different samples were prepared: with air-drying treatment or solar-drying treatment, or without treatment. Untreated and dried samples were extracted using the MAE, UAE, and Soxhlet extraction with water, water:ethanol (1:1), and ethanol as solvents to recover bioactive compounds with high antiradical properties. The drying of grape pomace plays an important role not only in the reduction of the volume of winery waste, but also in the effectiveness of the recovery of bioactive compounds. The highest recovery was in the case of UAE with the use of water:ethanol (1:1) for air-dried grape pomace and solar-dried grape pomace (438,984 and 258,663 ppm gallic acid equivalent in dry extracts, respectively). Moreover, for the untreated grape pomace, the most effective was MAE with the use of water:ethanol (1:1) (219,228 ppm gallic acid equivalent in dry extract).

In the light of recent studies, researchers should focus on extraction with the use of eutectic solvents. Eutectic (low-melting-point) mixtures are liquids formed from natural substances. For example, organic acids (e.g., citric acid), polyols (e.g., glucose), and salts (e.g., choline

chloride, sodium citrate) belong to this group. Currently, these liquids attract attention as green extraction media because they are characterized by reduced toxicity, ease of preparation and use, and low cost [112]. UAE of by-products from agriculture was conducted with eutectic solvents as 90% (v/v) aqueous solutions of glycerol:choline chloride (molar ratio of 3:1), glycerol:sodium acetate (3:1), and glycerol:sodium-potassium tartrate:water (5:1:4) [113]. Water and 60% (v/v) aqueous ethanol were used as control solvents. The results showed that the recovery of grape pomace polyphenols with the use of glycerol:choline chloride had the highest efficiency, which was comparable to that of recovery with aqueous ethanol.

The recovery of phenolic compounds from wine residues was also conducted with the sequential processes of centrifugation, MF, UF, and nanofiltration (NF) [114]. This combination allowed the recovery of phenolic products with 45% content of gallic acid equivalents. The antioxidant capacity of the wine residues was analyzed with a 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method, and 2 g of Trolox was obtained. This result indicated that the obtained polyphenols could be used as antioxidant products due to their high antiradical properties. The total phenolics and COD were eliminated at the level of 85.1% and 91.8%, respectively. Thus, these processes could serve as a pre-treatment stage for wine residues.

Recovery of Polysaccharides

Polysaccharides are low-cost materials and have many applications mainly in the food industry. Some function as emulsifiers and surface active agents, and many are used as simple thickeners. They can be classified as viscosity-increasing agents and as gelling agents. Some polysaccharides are used to the preparation of dextran and pullulan and rare sugars [115].

Giacobbo et al. [65] recovered polyphenols and polysaccharides from winery wastewater by sedimentation and UF processes. The sedimentation conditions were optimized by changing the pH from 3.8 to 8.0, while UF was optimized by changing the transmembrane pressure from 0.5 to 4.0 bar and the feed velocity from 0.44 to 0.87 m/s. This process reduced the TOC content by 56.6% whereas the polyphenols and polysaccharides in the retentate have been concentrated by 6 and 5 times, respectively. The concentrations of total polyphenols and total polysaccharides were 7,014 mg/L and 23,892 mg/L, respectively.

Giacobbo et al. [116] investigated the use of aqueous extraction and MF for recovery of polysaccharides and polyphenols from effluents from the first racking of red wine. The most effective combination involved fiftyfold dilution of the solution and MF, which produced a clear permeate. With this combination, 1 g of polysaccharides and 1 g of polyphenols from 1 liter of wastewater were recovered.

Recovery of VFAs

In addition to biogas production, recovery of VFAs from industrial wastewater is another method of making wastewater treatment economically attractive. These substances can be produced by elimination of the methane-forming phase of anaerobic digestion/fermentation [117]. VFAs are linear short chain (C2-C6) carboxylic acids and functional molecules, which serve as precursors for the sustainable production of added value chemicals (alcohols and aldehydes), polymers and biofuels [118, 119], and polyhydroxyalkanoates [120].

Although VFAs can be produced by processing petrochemicals, this leads to serious negative health and environmental effects. Greenhouse gas emissions from acetic acid

Table 4 Overview of methods of recovery of polyphenols, polysaccharides, and VFAs

Method	Additional conditions	Type of value-added product	Concentration of value-added product	Reference
Extraction using 53% ethanol aqueous assisted by microwave (100–600 W)	–	Polyphenols	40.35 mg GAE/g dry pomace	[127]
Extraction using 53% ethanol aqueous assisted by ultrasound (20–60 °C)	-	Polyphenols	33.88 mg GAE/g dry pomace	
Extraction using 53% ethanol aqueous assisted by ultrasound (58 °C)	Pretreatment with cellulase	Polyphenols	44.58 mg GAE/g dry pomace	
Ultrafiltration	Aqueous extraction and fiftyfold dilution	Polyphenols Polysaccharides	26.4 mg/L of waste 17.3 mg/L of waste	[128]
Microfiltration	Pre-sedimentation	Polysaccharides	17.89 mg/L	[129]
Anaerobic membrane bioreactor	pH 9; transmembrane pressure 0.5–4.0 bar	Polysaccharides	26.2 mg/L	[130]
	Feed velocity 0.44–0.87 m/s	VFAs	24.0 mg/L	
Batch anaerobic acidogenic wet-process fermentation	–	VFAs	22.20 mg/L (acetic 15.50 mg/L and butyric 4.30 mg/L)	[120]
Extraction using 80% methanol aqueous	–	Polyphenols	28.13 mg/g dry matter	
Supercritical carbon dioxide (SC-CO_2)	Containing 10% ethanol aqueous	Polyphenols	25.27 mg/g dry matter	

production in the petrochemical industry, calculated as CO_2 equivalent, are 3.3 t/t [121]. Distillery stillage contains sufficient carbon to produce VFAs via acidogenic fermentation, and it is thus a potentially attractive substrate for production of these valuable chemicals. An advantage of acidogenic fermentation is that it can be carried out by mixed cultures, which means that the substrate does not need to be sterilized [122]. To conduct acidogenic fermentation for the production of VFAs, the methanogenic stage of anaerobic digestion can be specifically inhibited by reducing the pH and the solids retention times [122]. The optimum pH for acidogenesis with distillery stillage ranges from 5.5 to 6.5, whereas a pH of 5.0 or less favors the production of alcohol, and a pH of 6.5 or higher favors biogas (methane) production [123]. Therefore, pH must be controlled with great care. The composition of the VFAs that are produced is also substantially affected by retention time [123, 124]; a retention time of 10–12 h has been reported to maximize acid production [125]. Martinez et al. [120] used acidic anaerobic fermentation of red grapes for VFA production. With the use of a batch anaerobic acidogenic wet process, it was possible to recover VFAs from the liquid phase. This process lasted for 16 days, after which 22.2 g/L of total VFAs was obtained; acetic (15.5 g/L) and butyric (4.3 g/L) acids predominated.

Zacharof and Lovitt [126] used membranes to recover VFAs from distillery stillage. MF was carried out to remove solid particles. In MF, 20.74% of the total solid content had been removed (from 15.13 to 11.99 g/L). After this pre-treatment, NF enabled recovery of acetate (53.94 mM) and butyrate (28.38 mM).

Table 4 presents the overview of methods and conditions of recovery of polyphenols, polysaccharides, and VFAs.

Challenges in Recovery of Valuable Products from Distillery Stillage

The approach of conversion of organic wastewater, such as distillery stillage, into valuable materials and energy, defines the biorefining concept as one of the elements of sustainable development. This so called waste bio-refinery is in fact a method of waste valorization [21]. However, development of the valorization technology requires the optimization of recovery techniques. So far, distillery stillage has not been tested intensively in terms of the possibility of recovery of polyphenols and other compounds with antioxidant activity. However, based on the recovery results from other wastewater like winery effluents, the recovery techniques will be developed. Currently, attention is being paid to searching for universal, non-toxic, and biodegradable solvents, particularly because natural bioactive compounds can be used in the pharmaceutical, agrochemical, nutraceutical, and cosmetic industry. In this regard, the use of deep eutectic solvents that contain two or more substances started to be tested. They include choline chloride with urea, carbohydrate with urea and chloride salts, decanoic acid with ammonium salts, choline chloride with oxalic acid, etc. Melting point of these solvents is lower than that of single substances and they can replace many conventionally used hazardous solvents in the extraction process [131]. It is known that the composition of eutectic solvents and thus their viscosity, surface tension, and polarity affect the extraction of bioactive compounds, because they influence mass transfer and diffusivity [132, 133]; however, such investigations for distillery residue are still in the future perspective. Studies on these solvents should focus on the selectivity, purity, and stability of the recovered bioactive compounds. The development of recovery using deep eutectic solvents on an industrial scale involves also the possibility of their recycling, which will reduce the process cost.

Conclusions

Expansion of distillery industry in the world made it necessary to develop effective technologies for the treatment of distillery stillage. Due to the presence of difficult-to-degrade compounds, physico-chemical, biological, and thermal treatment processes, such as aerobic and anaerobic biological treatment, coagulation/flocculation, electrocoagulation, adsorption, advanced oxidation, membrane processes, and evaporation/combustion, have been explored during recent years to address the demands of environmental regulations. It has been documented that for effective degradation of organic matter, particularly in terms of water reuse, a comprehensive treatment that involves several sequential technologies should be employed. Apart from this, much attention is paid to development of membrane treatment and particularly to exploring new membrane materials that will decrease membrane fouling, thus giving optimal permeate flux, to optimizing energy demand and to valorization of retentate.

As it was presented through this review, distillery stillage is a source of valuable substances such as polyphenols, polysaccharides, and VFAs. The methods of their recovery, according to the biorefinery concept, have been overviewed, indicating the need to focus on the selectivity, purity, and stability of the recovered compounds. However, in the light of recent studies, future research should include the searching for biodegradable, reusable, and non-toxic solvents for effective extraction of these bioactive compounds. Hence, the investigations of the use of deep eutectic solvents for valorization of distillery stillage still remains a considerable challenge.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Recovery of polyphenols from distillery stillage by microwave-assisted, ultrasound-assisted and conventional solid–liquid extraction

Wioleta Mikucka[1]✉, Magdalena Zielinska[1], Katarzyna Bulkowska[1] & Izabela Witonska[2]

Recovery of bioactive compounds from distillery waste could be an option for valorizing this waste. This study investigated how the extraction of polyphenols (which have antioxidant activity) from distillery stillage was affected by solvent type and concentration, extraction time, and method of extraction (conventional solid–liquid extraction, CSLE; ultrasound-assisted extraction, UAE; microwave-assisted extraction, MAE). Although recovery was similar with UAE and MAE, 3 min MAE with 80% ethanol and 80% methanol produced the highest yields of total phenolic content (TPC), total flavonoid content (TFC) and phenolic acids. With CSLE, TPC was 2.1–1.8-times lower than with MAE and 1.7–1.4-times lower than with UAE. Increasing the solvent concentration to 100% significantly decreased recovery. Six phenolic acids were recovered (ferulic and p-coumaric acid predominated), which were present mainly in the free form. There was a significant positive correlation between antioxidant activity, as measured with three methods (one based on the hydrogen atom transfer and two based on single electron transfer mechanisms), and phenolic acid content. With MAE and UAE, polyphenols were recovered more efficiently, with 2.1 times and 1.5 times higher antioxidant activity, and with 15 times and 9 times shorter extraction times, respectively, than with CSLE; thus, they can be considered "green" alternatives to CSLE.

The distillery industry makes large contributions to the global economy; however, distilleries generate waste (termed distillery stillage), which contributes significantly to environmental pollution. For every liter of alcohol produced, ca. 8–15 L of by-products are generated which have a low pH and a dark brown color and contain large concentrations of hardly degradable organics and nitrogen[1]. Environmental regulations and increasing understanding of the negative effects of waste discharges have made both treatment and valorization of distillery by-products to produce value-added compounds reasonable options for distillery stillage management.

Distillery stillage contains bioactive products such as polyphenols[2] with beneficial effects on health due to their antioxidant, antimicrobial and anti-inflammatory properties. Therefore, they can be used in the pharmaceutical, cosmetic, and food industries[3]. On the other hand, at concentrations above 1 g/m^3, polyphenols may inhibit methanogenesis during anaerobic processing of waste[4]. Therefore, more attention should be paid to the extraction of these bioactive compounds from distillery waste and to limiting their emissions into the natural environment.

Polyphenols differ in terms of chemical structure, which is a very important aspect when choosing solvents and determining the extraction conditions. The most common polyphenols are flavonoids and phenolic acids[5]. The bound forms of phenolic acids account for 80–95% of their total amount and they have linkages with cell wall polymers. To release them from the cell wall matrix, acidic and basic hydrolyses have been performed[3]. In extraction, 80% ethanol was used to recover polyphenols from cereals[6]. As a result, five phenolic acids were

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obtained with concentrations decreasing in this order: syringic > sinapic > ferulic > p-coumaric > coffee. When extraction was preceded by alkaline hydrolysis, seven acids were obtained with concentrations decreasing in the following order: ferulic > sinapic > vanillic > p-hydroxybenzoic > p-coumaric > syringic > coffee. In another study, the amount of gallic acid obtained after acidic hydrolysis of grape skins without ultrasound irradiation was 4.5 times higher than after extraction without hydrolysis but supported by ultrasounds[7]. With ultrasonic treatment, they recovered the following acids: gallic > p-coumaric > ellagic > vanillic, whereas after hydrolysis, they obtained acids: gallic > p-hydroxybenzoic > p-coumaric > syringic > ellagic > vanillic. Hence, the operational conditions of extraction strongly affect the composition of polyphenols.

Conventional solid–liquid extraction (CSLE) uses organic solvents due to their high selectivity for polar compounds[8]. Nevertheless, the search for new extraction techniques has resulted in the development of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) for polyphenol recovery[9]. However, polyphenols may be degraded at high temperatures[10] and both the extraction yield and the antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarities[11]. Additionally, phenolic compounds can form strong bonds with lignin[2]. Therefore, these extraction techniques should be optimized in terms of types of solvents and operational conditions for each type of waste material.

Antioxidant activity of polyphenols can be monitored by several methods, which are based on the inhibitory action of extracts on free radical activity of compounds used in the antioxidant tests. These tests include the use of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and iron-reducing antioxidants (FRAP). In general, the total activity of a certain antioxidant can be assessed in terms of hydrogen atom transfer (HAT) or single electron transfer (SET)[12]. In most situations, these two reactions occur simultaneously, and the mechanism is determined by the structure and solubility of the antioxidant, the partition coefficient, and the polarity of the solvent. Because diverse mechanisms are involved in the antioxidant action, to obtain a clear understanding of the relationship between the structure and the activity of the antioxidant, tests based on the HAT and SET are used. ABTS test is based on HAT, whereas DPPH and FRAP tests – on SET mechanisms. Despite the widespread use of these tests, agreement between them depends on the substrate being used. For example, Ou et al.[13] showed no correlation between FRAP and ABTS results for vegetable samples, while in the case of blueberry fruit these results were highly correlated[14]. Likewise, Awika et al.[12] showed a strong correlation between ABTS, DPPH, and FRAP results in sorghum and its products. Since the extraction yield and antioxidant activity of polyphenols are strongly dependent on the substrate from which the polyphenols are recovered, such studies are required for each substrate[15]. Because the proportions of phenolic acids vary considerably depending on the type of substrate and the extraction conditions and may affect the antioxidant activity, the solvent should be selected not only for its extraction efficiency, but also regarding the antioxidative properties of the extracted compounds[8].

Up to now, recovery of polyphenols with simultaneous analysis of their antioxidant activity has been performed with cereals[6], and regarding wastes, with olive mill wastewaters, vinasses, apple pomace, orange peels, or grape marc[13,15,16]. Based on a literature review, it appears that there is a lack of information on the possibility of recovering polyphenols from distillery stillage. Hence, this study focused on the stillage and investigated the effect of extraction conditions, such as the type and concentration of solvent used, and the time of CSLE, UAE and MAE, on (i) the total polyphenol content (TPC) and total flavonoid content (TFC), (ii) the content and types of the major free and bound phenolic acids in the extracts, and (iii) the antioxidant activity of polyphenols using the ABTS, FRAP and DPPH tests.

Materials and methods

Sampling and preparation. Distillery stillage came from a company in north-east Poland that produces concentrated unpurified ethyl alcohol from cereals (wheat and rye). The stillage was characterized by: 43,600–50,400 mg COD/L, 4,345 ± 5 mg N_{tot}/L, 280 ± 2 mg P_{tot}/L, and 789 ± 3 mg CH_3COOH/L. After transporting to the lab, the stillage was frozen and then freeze-dried (STERIS Lyovac GT2 freeze dryer with Leybold Trivac vacuum pump).

Extraction of polyphenols. For freeze-dried stillage samples, three extraction methods were tested: CSLE, UAE and MAE with the use of different solvents and times of extraction. CSLE, UAE and MAE were carried out with aqueous solutions of ethanol (E) and methanol (M) at concentrations of 60–100%. In the series abbreviations, the values after CSLE, UAE and MAE mean the extraction time, while the values after E and M mean the solvent concentration. For each extraction method, the ratio of freeze-dried distillery stillage to solvents was 1:30 (w/v). CSLE was carried out under shaking conditions for 15, 30, 45, 60, and 90 min, with three extraction cycles, at room temperature. For UAE, an ultrasonic bath (InterSonic IS–5,5) with a frequency of 25 kHz was used. The samples were sonicated for 3, 5, 10, 15, and 20 min, at room temperature. MAE was performed using a microwave oven (MARSXpress 240/50, CEM) for 1, 3, 5, 8, and 10 min at 400 W, at 50 °C. After extraction, samples were centrifuged in a 5810R Eppendorf centrifuge for 10 min at 10,000 rpm. In supernatants (extracts), electrical conductivity (EC) (Multi-Range HI8733 conductivity meter), pH (pH meter, HANNA Instruments HI 221), and surface tension (ST) (K100C tensiometer) were measured at room temperature.

Quantification of TPC and TFC in the extracts. The TPC was measured using the spectrophotometric method with Folin-Ciocalteu (F–C) reagent, according to Singletion et al.[17] with modifications, and then expressed as Gallic Acid Equivalents (mg GAE/g DM).

The TFC was measured by the aluminum chloride colorimetric method, according to Quettier-Deleu et al.[18] with modifications, and then expressed as Quercetin Equivalents (mg QUE/g DM).

Determination of free-soluble and bound-insoluble phenolic acid content by HPLC. To determine the content of free phenolic acids, the supernatant samples were evaporated to dryness in a rotary vacuum evaporator (Vacuum Rotavapor R-210, BUCHI) at below 50 °C. Extraction of free and total phenolic acids was conducted according to the method of Yang et al.[19] with minor modifications. To determine the free phenolic acids, 20 mL of distilled water (acidified to pH 2) was added to dry extracts to dissolve the precipitate after drying. To determine total phenolic acids, hydrolysis was performed. 1 g of the freeze-dried sample was mixed with 20 mL of 2 M NaOH for 4 h. The samples were acidified to pH 2 with 6 M HCl and centrifuged for 10 min at 10,000 rpm. The supernatants with free and total phenolic acids were extracted four times using 20 mL of diethyl ether. The diethyl ether extracts were dried by a rotary vacuum evaporator and re-dissolved in 1 mL of methanol before being subjected to chromatographic separation. Phenolic acids were determined according to the method described by Chiremba et al.[20] with modifications. Chromatographic separation was performed by a HPLC (Varian, Australia) fitted with a UV–Vis detector equipped with a Supelcosil C18 column (150 mm × 4.6 mm, 5 µm) with the column flow rate set to 1 mL/min, and the temperature set to 35 °C. The mobile elution phase was acetonitrile/formic acid (99.85/0.15, v/v) (eluent A) and water/formic acid (99.85/0.15, v/v) (eluent B). Phenolic acids were separated for 42 min at a gradient elution flow rate of 1 mL/min with the following multistep gradient: 0–18 min, 1–96% B; 18–35 min, 96–82% B; 35–40 min, 82–75% B. The analysis used wavelengths of 260 nm (p-OH-benzoic, vanillic, syringic acids) at 0–16.50 min and 320 nm (p-coumaric, ferulic, sinapic acids) till the end. All phenolic acid standards were prepared at a concentration of 0.1 mg/mL. Phenolic acids were identified based on the standard curves of the absorption spectra of the reference phenolic acids.

All extraction procedures were first performed with samples of distillery stillage that were spiked with phenolic acid standards. For this procedure, phenolic-acid standard solutions of 0.1 mg/mL were dissolved in acetonitrile, and then 1 mL of these solutions was added to 1 g of freeze-dried stillage. The samples were left for 24 h in the dark.

Determination of antioxidant capacity. To measure the antioxidant activity of the extracts as their capacity to scavenge free radicals based on the HAT and SET mechanisms, ABTS, DPPH, and FRAP tests were used. The ABTS activity was determined as described by Re et al.[21] with modifications and expressed as Trolox Equivalents (µmol TE/g DM). The DPPH activity was determined according to Moure et al.[15] and expressed as Trolox Equivalents (µmol TE/g DM). The FRAP activity was determined according to Benzie and Strain[22] and expressed as $FeSO_4$ equivalents (µmol $FeSO_4$/g DM).

Method validation. The performance of the procedure for identifying phenolic acids in the extracts was assessed using the following indicators: linearity, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

To determine linearity, a standard curve was generated by diluting standard solutions. From the peak-concentration diagram, the coefficients of determination (R^2) were calculated using linear regression.

To determine the method accuracy, recovery tests were carried out using spiked samples with three concentrations of standard solution (low, medium, high). The recovery efficiency (%) was calculated according to the following equation: $R = [(A1 - A2)/A3] \times 100$, where A1 is the concentration of compounds recovered from the spiked sample, A2 is the concentration of compounds recovered from the sample without spiking, and A3 is the concentration of compounds in the spiked samples.

The LOD and LOQ were calculated based on the analytical curve by selecting appropriate signal-to-noise ratios (3:1 and 10:1, respectively) and using the standard deviation of the response (σ) and the slope of the curve (S). The equations used were as follows: $LOD = [(3 \times \sigma)/S]$ and $LOQ = [(10 \times \sigma)/S]$.

Data analyses. TFC, TPC, concentrations of phenolic acids, ABTS, FRAP, and DPPH were expressed as mean ± standard deviation. All extractions were performed in duplicate, and analyses were performed in triplicate. Spearman's rank correlation coefficient (r_s) was used to quantify the relationships between the polyphenol content, antioxidant activities, the physicochemical characteristics of solvents and the extraction type. The correlation matrix was visualized using a correlogram with RStudio Version 1.2.1335 using the "corrplot" package. For statistical analysis, STATISTICA 13.1 (StatSoft) was used, with $p \leq 0.05$ defined as significant.

Results and discussion

Qualitative assessment of extraction correctness using the standard addition method. The recovery efficiencies of phenolic acids ranged from 52 to 99% (Table 1SM) and decreased in this order: MAE > UAE > CSLE. Using the standard addition method, similar results to those presented in this study were obtained by Robbins and Bean[23], who reported 69–97% efficiency of polyphenols recovery from wine waste. The efficiency of phenolic acid extraction from non-hydrolyzed and hydrolyzed beer samples was 95.4–104.0% and 94.0–102.9%, respectively[24]. Hence, the present results of the standard addition method confirmed the correctness of the adopted extraction methodology and the procedures, which were performed for quantification of phenolic acids.

Validation of HPLC method. A wide linear range of standard solutions was used due to the lack of literature reports on the expected phenolic acid concentrations recovered from distillery stillage and the concentration differences between the detected acids (Table 1). The average recoveries were 87.2–96.4% (p-OH benzoic), 95.6–101.7% (vanillic), 79.1–87.7% (syringic), 96.4–103.7% (p-coumaric), 103.7–105.7% (ferulic) and 93.2–102.8% (sinapic). These values were well within acceptable limits (70–110%)[25], indicating the appropriate-

Type of acids	Retention time (min)	λ (nm)	Linear range (µg/L)	R^2	Accuracy level (%)			slope	intercept	LOD (µg/L)	LOQ (µg/L)
					Low	Medium	High				
p-OH benzoic	10.42	260	1–800	0.997	96.4	94.1	87.2	0.0727	0.2475	0.61	2.02
vanillic	12.92	260	1–1000	0.998	101.1	101.7	95.6	0.0459	0.0686	0.95	3.16
syringic	14.42	260	3–800	0.998	84.3	87.7	79.1	0.0205	0.0389	1.79	5.99
p-coumaric	18.29	320	0.7–1000	0.991	103.7	102.4	96.4	0.0804	0.2327	0.49	1.63
ferulic	20.51	320	1–1000	0.999	104.9	103.7	105.7	0.0617	0.0989	0.66	2.19
sinapic	21.08	320	0.5–1000	0.999	95.7	93.2	102.8	0.1032	0.0442	0.43	1.42

Table 1. Validation parameters.

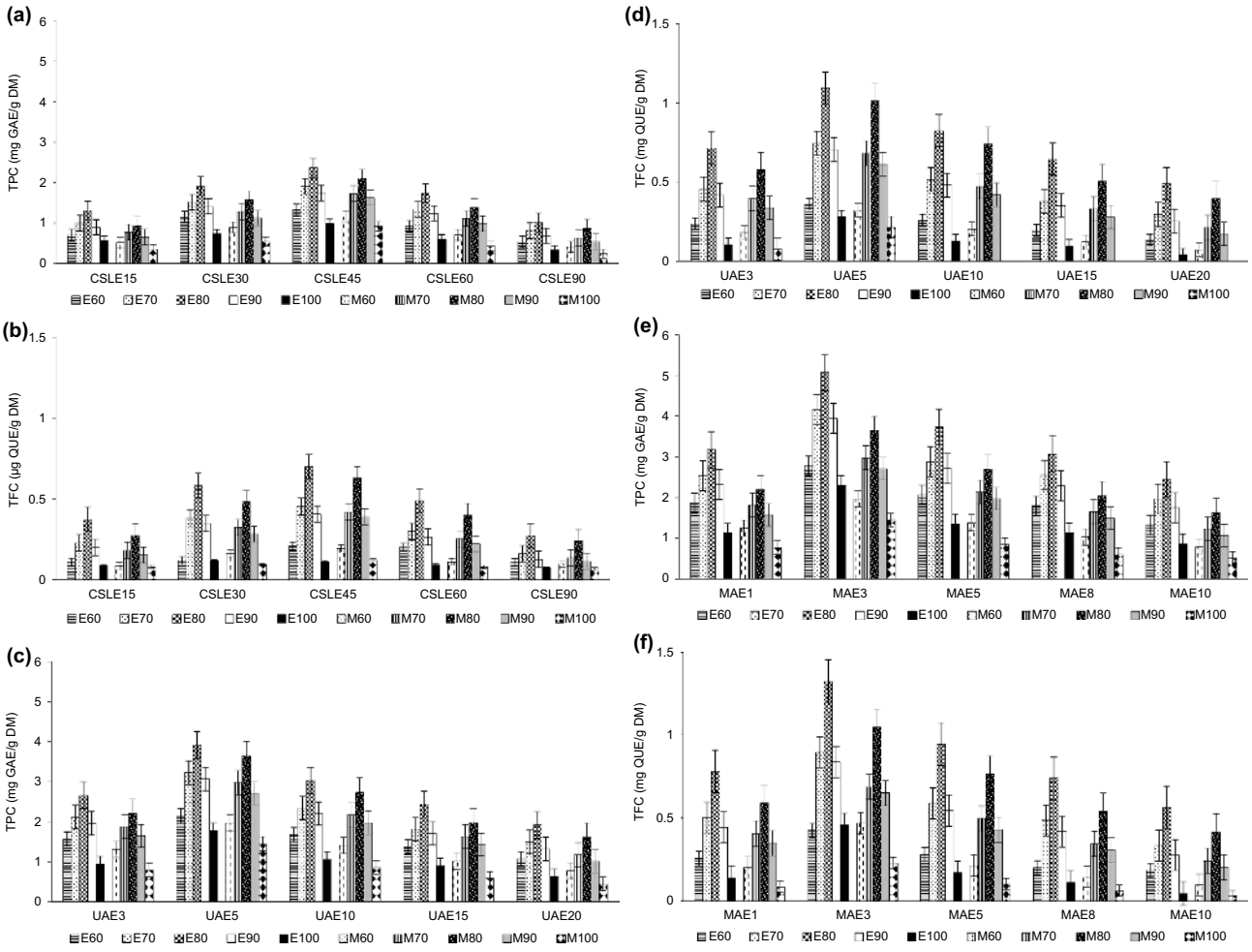


Figure 1. TPC and TFC in distillery stillage extracts obtained with CSLE (**a**,**b**), UAE (**c**,**d**), and MAE (**e**,**f**) for varying extraction time.

ness of the methodology; recovery percentages greater than 100% are explained by overlapping sample matrices. The LOD and LOQ ranged from 0.43 to 1.79 µg/L and from 1.42 to 5.99 µg/L, respectively.

TPC and TFC in distillery stillage extracts. TPC and TFC were recovered most efficiently with MAE, followed by UAE, and then CSLE (Fig. 1). TPC was highest when MAE was applied for 3 min with 80% ethanol or 80% methanol (5.07 ± 0.03 mg GAE/g DM or 3.65 ± 0.03 mg GAE/g DM, respectively) (Fig. 1e). With UAE, TPC was slightly lower than with MAE. Recovery with UAE was highest when it was applied for 5 min with 80% ethanol or 80% methanol (3.92 ± 0.02 mg GAE/g DM or 3.65 ± 0.02 mg GAE/g DM, respectively) (Fig. 1c). CSLE was the least efficient method for recovering TPC. The yields were 2.1–1.8-times lower than with MAE and 1.7–1.4-times lower than with UAE. Recovery with CSLE was most effective when it was applied for 45 min with 80% ethanol or 80% methanol (2.36 ± 0.01 mg GAE/g DM or 2.10 ± 0.01 mg GAE/g DM, respectively) (Fig. 1a).

The TFC recovered with MAE during the extraction times decreased in the following order: 3 min > 5 min > 1 min > 8 min > 10 min. With 3 min of MAE, 1.32 ± 0.01 mg QUE/g DM and 1.19 ± 0.02 mg QUE/g DM were obtained with 80% ethanol and 80% methanol, respectively (Fig. 1f). With UAE, the amount of TFC decreased in this order: 5 min > 10 min > 3 min > 15 min > 20 min. With 5 min of UAE, 1.14 ± 0.02 mg QUE/g DM and 1.02 ± 0.01 mg QUE/g DM were obtained with 80% ethanol and 80% methanol, respectively (Fig. 1d). With CSLE, the amount of TFC decreased in this order: 45 min > 30 min > 60 min > 15 min > 90 min. With 45 min of extraction, 0.71 ± 0.02 mg QUE/g DM and 0.63 ± 0.01 mg QUE/g DM were obtained with 80% ethanol and 80% methanol, respectively (Fig. 1b).

In the extraction methods, the shortest times of extraction were selected to be 15 min in CSLE, 3 min in UAE, and 1 min in MAE. Such short contact times are not used commonly to recover polyphenols. However, because the conditions of extraction depend on the sample type[26], the optimization of extraction time for not recognized so far distillery stillage was started from these times. Unexpectedly, the obtained yields were similar or higher than at the longest time (90 min in CSLE, 20 min in UAE, and 10 min in MAE). To conclude the effect of extraction time on polyphenol yield, TPC and TFC increased significantly when the time was lengthened from 1 to 3 min (MAE), from 3 to 5 min (UAE), or from 15 to 45 min (CSLE). Further extraction lengthening decreased TPC and TFC. Similarly, when compared to CSLE, the increase in extraction yield of TPC was 200% after 5 min and 100% after 15 min of UAE[27]. Although MAE and UAE results in a partial disruption of the polyphenol network into low molecular weight polyphenols, which allows for release the intracellular bioactive compounds in a short time[16,28], polyphenols may be degraded if they are exposed to microwaves and ultrasounds for too long. This is visible in the present study, in which the TPC and TFC in the extracts decreased when MAE and UAE exceeded 3 min or 5 min, respectively. These decreases may have been caused by the formation of hydrogen peroxide in polyphenol extraction[29] and the production of free hydroxyl radicals which degrade polyphenols, particularly in the presence of high water content when being exposed to ultrasound[30]. This hypothesis was tested in the present study in a preliminary experiment with known quantities of phenolic acids spiked to the stillage, which was conducted in all extraction variants using the same extraction conditions as in the main experiment. The content of phenolic acids in the extracts decreased when MAE and UAE exceeded 3 min or 5 min, respectively, which indicated degradation of phenolics with the same trend as in the main experiment. In addition, longer extraction times also increased exposure to oxygen and light, which degraded polyphenols[31]. On the other hand, increasing the time of MAE from 5 to 15 min did not change significantly the extracted TPC from spinach waste[32]; however, this relation was valid for a high percentage of ethanol and attributed to the higher solubility of less polar compounds in higher ethanol concentrations.

TPC and TFC were about 1.9–times higher with MAE and UAE than with CSLE because the ultrasounds and microwaves create a faster and stronger mixing effect than that with CSLE. This effect disrupts the cell structures and reduces external resistance, which increases the accessibility of the solvent to the internal particle structure, thus increasing extraction efficiency[33]. Due to temperature and pressure in MAE and UAE, the release of products of the Maillard reaction (during alcohol production) may have been increased. These substances would have reacted with the F–C reagent and aluminum chloride that are used in the TPC and TFC analyses, respectively[34], resulting in the higher measured TPC and TFC. In the present study, the TPC obtained with MAE and UAE was rather similar, as was the TFC obtained with these two methods. This is because the products of the Maillard reaction can only be present in soluble fractions. During the production of alcohol, cereals are fermented, mixed, and thermally treated, which increases the amount of soluble compounds. Most of these compounds remain in the production environment, while only a small part passes into the stillage. This corresponds to the effect of MAE and UAE on the TPC recovered from vegetable wastes without their previous fermentation. When recovering polyphenols from orange waste, MAE provided ca. fourfold higher extraction yield than UAE because of radiation-inducing rupture of the vegetal cells[32].

In the present study, the solvent type influenced the polyphenol recovery from distillery stillage (Fig. 1). The use of ethanol recovered more polyphenols than the use of methanol. These results cannot be explained by differences in the presence of oxygen, which may degrade polyphenols, in the extraction solvents. Although the solubility of oxygen in ethanol is higher than in methanol[35], the solubility difference is not high enough to affect the efficiency of TPC extraction in the present study. It has been found that ethanol recovers flavonoids more efficiently than methanol because it interacts with compounds via non-covalent interactions and promotes their diffusion into solution[36]. On the other hand, Falleh et al.[37] observed that TPC was higher in the methanolic extract than in the ethanolic extract. These contradictory results can be explained by the fact that different types of polyphenols are extracted by the different solvents due to affinity differences resulting from the different polarities of a target compound and a solvent. Additionally, the TPC and TFC are determined by the substrate from which they are recovered. Various interactions between the components of the substrate and bioactive compounds can change the affinity of these compounds to specific solvents. Hence, determining the effect of a given solvent on the efficiency of polyphenol recovery is still a challenge due to the diversity of binding compounds in the matrix.

In the present study, 80% solutions of ethanol or methanol were most effective in TPC and TFC recovery, which may be due to the solubility of polar carbohydrates and glycosides of secondary metabolites in these solutions. In contrast, TPC and TFC recovery were lowest when 100% ethanol or methanol were used in CSLE, MAE, and UAE (Fig. 1). The fact that pure alcohol solutions were least effective for polyphenol recovery was related to changes in the polarity of the solvent solution. In general, extraction is improved by adding water to solvent, which increases solvent polarity, thus making it more similar to the polarity of phenolics. This similarity intensifies the molecular forces, thus increasing the solubility of the target compounds. This was particularly visible in MAE, in which the extraction yield was improved, as the absorption of microwave radiation is more efficient in polar media[26]. In the present study, the increase in extraction yield with the increasing percentage of ethanol or methanol to 80% and the decrease in the yield when the percentage was higher than 80% indicate that the extracted compounds were of a wide range of polarity. Similarly, when extracting polyphenols from the

olive pomace, increasing the ethanol percentage improved the recovery of less polar compounds and decreased the recovery of polar compounds[26]. In addition, because solvents with high polarity can extract compounds with a wide polarity, higher values of TPC in the present study when using solvents of percentages lower than 100% may result from the fact that non-phenolic polar compounds such as carbohydrates and proteins were dissolved during the extraction process, which resulted in increased extraction yields[38].

The addition of water to alcohol significantly lowered the ST of solvents. With 80% ethanol and methanol, the ST was 21% lower than that of the extracts obtained with pure alcohols. The reduction of the solvent ST increases the mass transfer of phenolic compounds and thus favors the extraction[10]. Additionally, increasing the water content in solvents helps to weaken the hydrogen bonds between the polyphenols and the cell matrix. This allows for better mass transfer of the compounds, and at the same time increases the solubility of the polyphenols, leading to an increase in the extraction yield[14]. However, in the present study, when the alcohol concentration was below 80%, there was a decrease in TPC. An increase in water content in the solvents could have favored the extraction of other compounds from the stillage which do not react with F–C reagent. For example, higher amounts of metal ions (Mn, Cu, Mg, Zn, Na, Ni, Fe, Co) were extracted from wheat seedlings to water extracts than to the ethanol extracts, whereas ethanol extracts had higher antioxidant properties due to polyphenol content than water extracts[39].

It was noted that the pH affected the TPC and TFC. The extracts obtained with CSLE, UAE, and MAE were acidic (4.16–5.32 pH). In the case of the hydrolysis process, the pH of the reaction mixture was about 2. Acidic conditions hydrolyze cell walls, contributing to the release of phenolics[40]. Our results are consistent with those of other studies, which have reported that lower pH values improve polyphenol extraction[41]. In general, the pH affects the stability and solubility of compounds[40], which in turn, affects extraction efficiency. More specifically, the OH groups in the phenolic compounds make them particularly susceptible to pH changes. In the present study, acidic conditions could have been particularly important in protection of polyphenols from oxidative degradation in UAE. Acids produce hydrogen ions that stabilize free radicals that may be generated during ultrasonication[42].

It should be taken into consideration that the F–C reagent is not specific for TPC determination. This reagent can also be reduced by other compounds that may be present abundantly in plant extracts (reducing sugars, ascorbic acid, proteins), distorting the results of TPC[43]. In this sense, measurement of phenols by the F–C method could possibly give too high an estimate of phenolic content. Andres et al.[44] found that F–C method indicated the phenolic content of 5.42 mg GAE/g in brewer's spent grain. However, HPLC indicated that the phenolic acid content was substantially lower: 178 µg/g. It was indicated that TPC ranges 60–70% of total extractable compounds[27]. Therefore, the polyphenol compounds were separated and quantified by HPLC in the present study.

Phenolic acid content in distillery stillage extracts. In HPLC, high-quality peak shapes and good separation were obtained, along with minimal baseline variability (Fig. 1SM). In all extracts, six phenolic acids were identified: p-OH benzoic, p-coumaric, vanillic, syringic, sinapic and ferulic. Their concentrations in the most effective extraction variants are shown in Fig. 2; in the other variants—in Fig. 2SM. Ethanol extraction with resulted in a higher content of free phenolic acids than methanol extraction. Both solvents performed more effectively when used in aqueous solutions than in pure form, which was less selective for phenolic acids.

Recovery of free phenolic acids was highest when using 80% ethanol or methanol during MAE for 3 min, with total values of 4.02 ± 0.05 µg/g DM and 3.51 ± 0.04 µg/g DM, respectively (Fig. 2c,f). With UAE, the concentration of phenolic acids was lower, amounting to 2.67 ± 0.04 µg/g DM or 2.49 ± 0.02 µg/g DM for 5-min extraction with 80% ethanol or methanol, respectively (Fig. 2b,e). CSLE was the least effective method for the recovery of phenolic acids (Fig. 2a,d). When using the same solvent at the same concentration, much higher concentrations of phenolic acids were recovered during 3 min of MAE and 5 min of UAE than during 45 min of CSLE. Due to higher extraction selectivity of MAE and UAE[45], these extraction times gave an increase in extraction yields by 221% (MAE) and 111% (UAE) with ethanol and by 182% (MAE) and 97% (UAE) with methanol, as compared to CSLE (Fig. 3).

So far, no research has been conducted on the phenolic acid recovery from distillery stillage, which comes from cereal processing. Data are available for the overall content of phenolic acids in cereals (e.g. in wheat: 177–257 µg/g DM[46], 867.38 µg/g DM[34], or 2612.38 µg/g DM[38]), or in wholemeal flours (4.63 to 7.24 µg/g DM[47]). Treatment and fermentation of the cereals used for alcohol production affect the content of recovered phenolic acids. For example, with spelt and rye breads, sourdough fermentation increased the accumulation of free phenolic acids, while with breads made of common wheat, this type of fermentation reduced their accumulation. Regardless of the fermentation type, free benzoic acids were preferentially accumulated in breads, especially p-OH benzoic and vanillic acids. Ferulic acid is the most abundant acid in maize, rice, wheat, buckwheat, millet, sorghum, rye, and barley[3,20]. Even though Shahidi and Naczk[5] found that most cereals and cereal products are rich in hydroxycinnamic acids, such as ferulic acid and p-coumaric acid, they reported that concentrations varied, depending on the source from which they were extracted.

In the present study, ferulic and p-coumaric acids predominated, accounting for 42% to 48% and 23% to 25% of all phenolic acids detected, respectively, depending on the extraction variant employed (Fig. 2). Next, vanillic and sinapic acids accounted for approx. 10–12% and 5–8% of this fraction, respectively. The sum of benzoic acids (p-OH benzoic and syringic) in the stillage was approx. 5–7%. This is similar to what was reported in other studies involving wheat, rye and sorghum, as well as flour and bread produced from these cereals, in which the content of ferulic, p-coumaric and vanillic acids was highest[48]. The phenolic acids that were detected in the stillage extracts in the present study are considered having some properties that are useful for the food, pharmaceutical and cosmetic industries. Due to low toxicity, ferulic acid is widely used as a cross-linking agent in the preparation of food gels and edible films, as a skin protection agent and as an ingredient in sports nutrition. P-coumaric

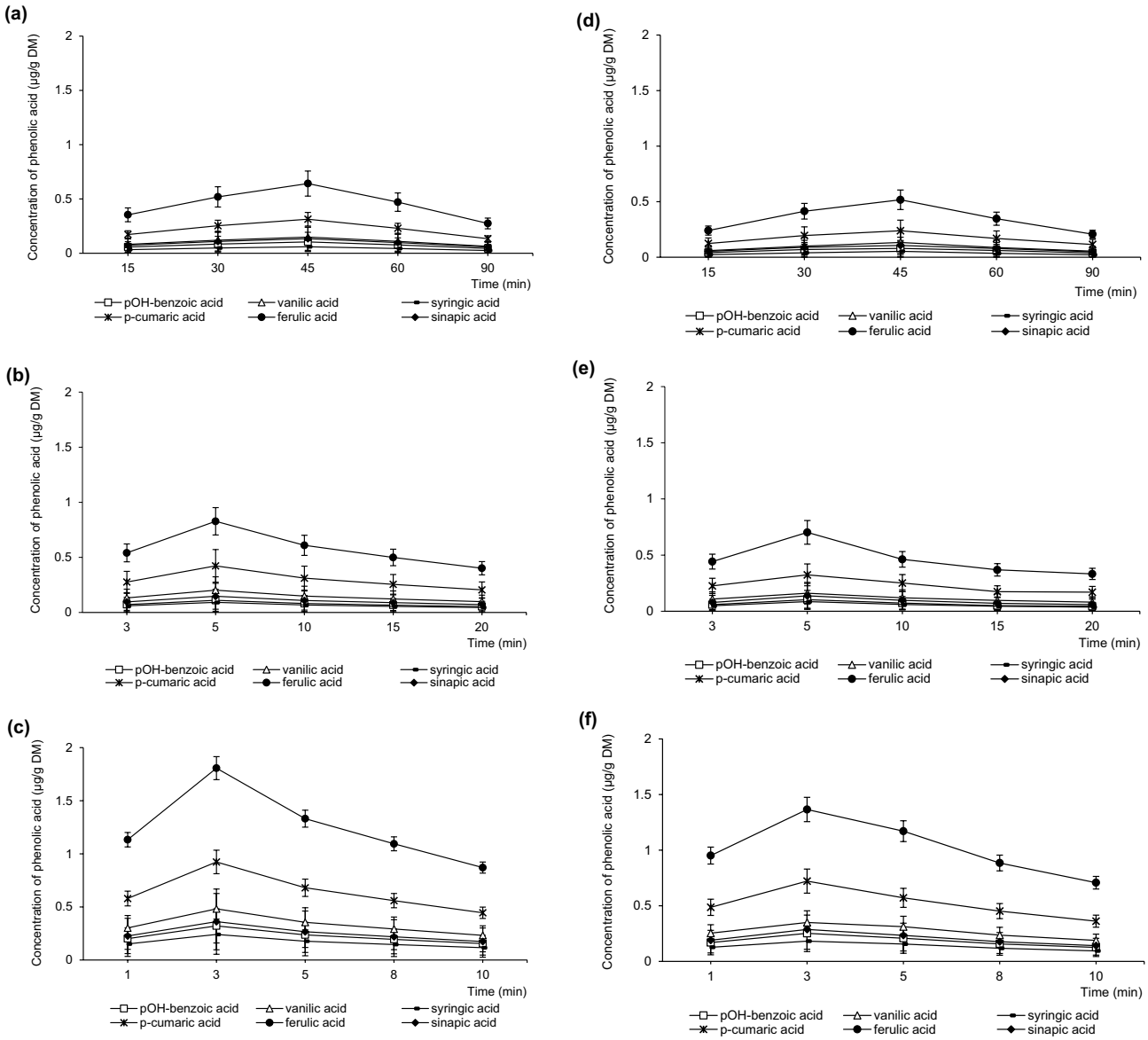


Figure 2. Concentrations of free phenolic acids in extracts obtained with (**a**) CSLE E80, (**b**) UAE E80, (**c**) MAE E80, (**d**) CSLE M80, (**e**) UAE M80, (**f**) MAE M80.

acid has a bactericidal effect[49]. Vanillic acid is used as a fragrance in the food and cosmetics industry[50]. Sinapic acid acts as a powerful oxidant scavenger, thus protecting cellular components[51].

Although many phenolic acids in cereal products are bound to cell wall materials, particularly polysaccharides, lignins and suberin, in the present study, only a small fraction of phenolic acids existed in a bound state. The concentration of free phenolic acids reached 4.02 ± 0.05 µg/g DM, and these acids constituted up to 92% of the total phenolic acids. The average concentration of total phenolic acids was 4.36 ± 0.60 µg/g DM. High contribution of free phenolic acids may be caused by the activity of phenolic acid esterases in fermentation and processing of cereals during the alcohol production process, when phenolic acids bound to the cell wall are released[47]. Additionally, during cereal processing, most of the phenolic acids pass into the product, while only a small part remains in the by-products. Moreover, heat treatment can cause changes in the phenolic content. In the present study, short duration of the supporting factor (UAE shorter than 5 min and MAE shorter than 3 min) resulted in the recovery of only a part of bound phenolic acids. However, with longer durations (UAE longer than 5 min and MAE longer than 3 min), the content of phenolic acids decreased in proportion with the increase in time (Fig. 2). These differences were most pronounced in the cases of ferulic acid and p-coumaric acid recovery: the respective amounts that were recovered were 2.23 and 1.94 times higher with 3-min MAE than with 10-min MAE, and 1.98 and 1.89 times higher with 5-min UAE than with 20-min UAE. Although it was reported that MAE or UAE improves the recovery, literature reports on the effect of time of exposition to this treatment are inconsistent. This is because these changes in phenolic acid content may result from the oxidative degradation of these acids, including enzymatic browning, the release of free acids from conjugated forms

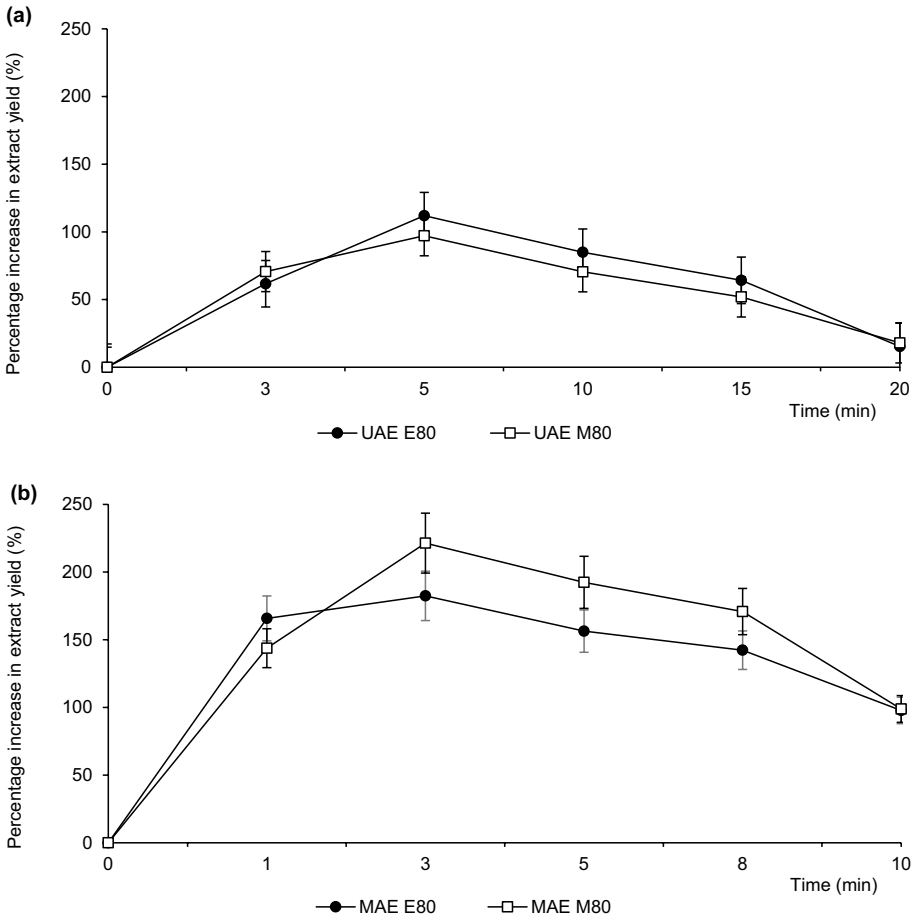


Figure 3. Effect of extraction time on the changes in yields of phenolic acids; (**a**) UAE, (**b**) MAE.

or the formation of phenolic substances with complex structures from related compounds, such as proteins, tannins and anthocyanins[52].

Antioxidant activity of polyphenols in distillery stillage extracts. The antioxidant activity of recovered phenolic compounds depends largely on the type, concentration, and polarity of the solvent used to recover them. Due to different mechanisms of reaction and conditions of these test, limitations of each test, and their ability to determine various types of antioxidants, the DPPH, ABTS and FRAP tests were used in the experiment. The antioxidant activities of the extracts obtained with MAE for 3 min with 80% ethanol were the highest, as indicated by the ABTS (26.73 ± 1.81 µmol TE/g DM), DPPH (5.57 ± 0.38 µmol TE/g DM) and FRAP (3.71 ± 0.26 µmol $FeSO_4$/g DM). When MAE was used for 3 min with 80% methanol, the extract had antioxidant activities that were somewhat lower (Table 2 and 2SM). The antioxidant activity was lowest with CSLE for 90 min with 100% ethanol and methanol. Higher antioxidant activity of ethanolic extracts than that of methanolic extracts corresponded with the higher TPC in ethanolic extracts. This relation between TPC and antioxidant activity may result from the absence of any potential interfering (absorbing) species which do not have antioxidant activity[53]. However, this relation is dependent on type of substrate and target compounds. Falleh et al.[37] reported that although TPC was higher in the methanolic extract than in the ethanolic extract, the ethanolic extract had higher antioxidant activity.

In all extracts, the ABTS assay indicated significantly higher antioxidant activity than the DPPH assay, which was followed by the FRAP assay. The predominance of antioxidant activity shown by the ABTS and DPPH over that shown by the FRAP may be due to the predominance of phenolic compounds that are efficient at scavenging radicals and smaller quantity of non-phenolic compounds that may be able to reduce FRAP, account for the reducing power but are not so efficient at scavenging radicals[53]. Similarly, Yu et al.[46] reported that the ABTS assay indicated that the activity of wheat bread extract was 10–20-times higher than what the DPPH assay indicated. Smuda et al.[54] found that the ABTS test indicated higher activity in by-products from wheat, rice and corn than the FRAP test. One observation for this is the fact that each test has a different mechanism, based on HAT (ABTS) or SET (DPPH and FRAP). The extract characteristics, including EC and pH, substantially influences these mechanisms of antioxidant activity[55]. In the present study, the EC of the extracts decreased as the solvent concentration was increased. For example, the EC in the extracts obtained from MAE3 was 7.5, 7.4, 7.2, 7.1 and 7.0 S/m for 60, 70, 80, 90 and 100% ethanol, respectively. The ABTS, DPPH and FRAP test values were correlated

Type of extraction	Antioxidant assays			Type of extraction	Antioxidant assays			Type of extraction	Antioxidant assays		
	ABTS (µmol TE/g DM)	DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)		ABTS (µmol TE/g DM)	DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)		ABTS (µmol TE/g DM)	DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)
CSLE15 E80	8.46 ± 1.88	2.41 ± 0.37	1.56 ± 0.10	UAE3 E80	14.60 ± 1.22	4.16 ± 0.26	2.70 ± 0.22	MAE1 E80	24.26 ± 1.56	5.05 ± 0.28	3.37 ± 0.18
CSLE15 M80	8.19 ± 1.57	2.34 ± 0.31	1.50 ± 0.09	UAE3 M80	14.12 ± 1.34	3.67 ± 0.38	2.45 ± 0.17	MAE1 M80	17.64 ± 0.89	4.04 ± 0.22	2.53 ± 0.23
CSLE30 E80	9.44 ± 1.92	2.72 ± 0.35	1.85 ± 0.18	UAE5 E80	16.67 ± 1.10	4.54 ± 0.41	2.96 ± 0.28	MAE3 E80	26.73 ± 1.81	5.57 ± 0.38	3.71 ± 0.26
CSLE30 M80	9.07 ± 1.67	2.59 ± 0.24	1.71 ± 0.21	UAE5 M80	15.48 ± 1.09	4.34 ± 0.31	2.75 ± 0.21	MAE3 M80	25.33 ± 0.78	5.27 ± 0.31	3.52 ± 0.25
CSLE45 E80	10.84 ± 2.01	2.95 ± 0.21	1.93 ± 0.19	UAE10 E80	14.99 ± 1.42	4.32 ± 0.28	2.94 ± 0.26	MAE5 E80	27.54 ± 0.65	5.74 ± 0.29	3.83 ± 0.29
CSLE45 M80	10.07 ± 1.79	2.82 ± 0.29	1.79 ± 0.19	UAE10 M80	14.39 ± 1.27	4.11 ± 0.21	2.71 ± 0.17	MAE5 M80	23.22 ± 0.84	4.83 ± 0.17	3.22 ± 0.15
CSLE60 E80	8.25 ± 1.56	2.34 ± 0.26	1.58 ± 0.15	UAE15 E80	14.23 ± 1.58	4.03 ± 0.27	2.72 ± 0.16	MAE8 E80	23.52 ± 0.74	4.90 ± 0.24	3.27 ± 0.11
CSLE60 M80	7.97 ± 1.42	2.23 ± 0.18	1.49 ± 0.14	UAE15 M80	13.74 ± 1.44	3.84 ± 0.34	2.57 ± 0.11	MAE8 M80	22.24 ± 0.63	4.63 ± 0.29	3.09 ± 0.04
CSLE90 E80	7.01 ± 1.68	1.89 ± 0.35	1.41 ± 0.11	UAE20 E80	12.74 ± 0.96	3.44 ± 0.18	2.56 ± 0.07	MAE10 E80	21.22 ± 0.87	4.42 ± 0.27	2.95 ± 0.08
CSLE90 M80	6.52 ± 1.86	1.77 ± 0.38	1.28 ± 0.08	UAE20 M80	11.86 ± 1.03	3.22 ± 0.14	2.32 ± 0.02	MAE10 M80	19.17 ± 0.57	3.99 ± 0.38	2.66 ± 0.11

Table 2. Antioxidant activities of distillery stillage extracts obtained with 80% solvents.

with EC; however, irrespective of these EC changes, values of ABTS test were higher than values of DPPH and FRAP tests in each extract. Therefore, EC changes in the stillage extracts did not explain these differences.

A second and related explanation for the differences in antioxidant activity measured with the three assays is the acidic extraction environment. Electron transfer reactions depends on the ionization potential of the active functional group and thus depends on the pH. The acidic environment inhibits the deprotonation of functional groups of polyphenolic compounds, which results in a reduced ability to donate electrons because deprotonation generally only lowers the ionization potential (SET) and not the bond dissociation enthalpy (HAT). Hence, it can be concluded that electron transfer is not the dominant mechanism of the antioxidant activity of the bioactive compounds examined in this study. However, it is worth noting that the mechanism of antioxidant action may change as the pH value increases. Thus, in this study, the environment was not conducive to the deprotonation of polyphenolic compounds, so H• donation became easier than electron donation. Therefore, the values of antioxidative activity obtained with the DPPH and FRAP tests were significantly lower than the values obtained with the ABTS test.

The reason for significantly different values of the antioxidant activity tests results from their principles. From three tests examined in this study, the values of the ABTS test were the highest because this test is used to measure the antioxidant capacity of both hydrophobic and hydrophilic samples, ABTS reagent is soluble in both aqueous and organic solvents, and the reaction of ABTS with flavonoids produces products with stronger antioxidant properties and faster reacting with the radical than other phenolic compounds[32]. The DPPH test does not allow the determination of hydrophilic antioxidants. Regarding the FRAP test, some compounds with antioxidant properties do not show reducing capacity towards FRAP reagent, and the formed iron ions can participate in the radical production in the presence of H_2O_2, which also distorts the result. That is why the values of the FRAP test were the lowest in this study.

Correlations between TPC, TFC, phenolic acids, antioxidant activity and extraction conditions. Antioxidant activity (ABTS) was strongly correlated with TPC and TFC ($r_s \geq 0.95$), indicating that TPC and TFC were mainly responsible for the activity of the extracts (Fig. 4). Antioxidant activity (ABTS) was positively correlated with the contents of p-OH benzoic (0.96), vanillic (0.97), syringic (0.96), ferulic (0.95), p-coumaric (0.98) and sinapic acid (0.98). The antioxidant effect of these extracts is attributed to the strong free radical scavenging effect of their free hydroxyl groups. This study showed that phenolic acid content was significantly negatively correlated with the extraction time, pH and ST. Their content was also negatively correlated with the concentration of the solvent, although these correlations were not statistically significant.

The relationship between antioxidant activity and polyphenol content can be explained in many ways. The synergy between the antioxidants makes the activity depend not only on the phenolic acid content but also on the structure and interactions between them. This is the reason why extracts with similar concentrations of TPC, TFC, and phenolic acids may differ in their antioxidant activity. Numerous studies of the antioxidant properties of phenolic acids suggested that they are related to the chemical structure of acids, more precisely to the number of hydroxyl groups in the molecule and the degree of their esterification. Balasundram et al.[56] noted that the antioxidant activity of phenolic acids increases with the number of hydroxyl groups. In compounds with a single hydroxyl group, the antioxidant activity is further enhanced by the presence of one or two methoxy groups on the ring, which is why sinapic acid with two methoxy groups is more active than ferulic acid with one methoxy group, which in turn is more active than p-coumaric acid (containing one hydroxyl group)[57]. The absence of methoxy groups creates a destabilized radical that is not sufficiently stable for p-coumaric acid to be an effective scavenger. In the present study, it was observed that ferulic and p-coumaric acids dominated among the acids in each extract. Along with an increase in their concentration, the antioxidant activity also increased. Therefore, the highest antioxidant activities were observed in ethanol extracts from 3-min MAE. Thus, the results clearly indicate that ferulic and p-coumaric acids have a strong antioxidant effect. It has been shown that the presence of a single CH bond, a double CH bond and a single COOH bond in hydroxycinnamic acids (ferulic, p-coumaric

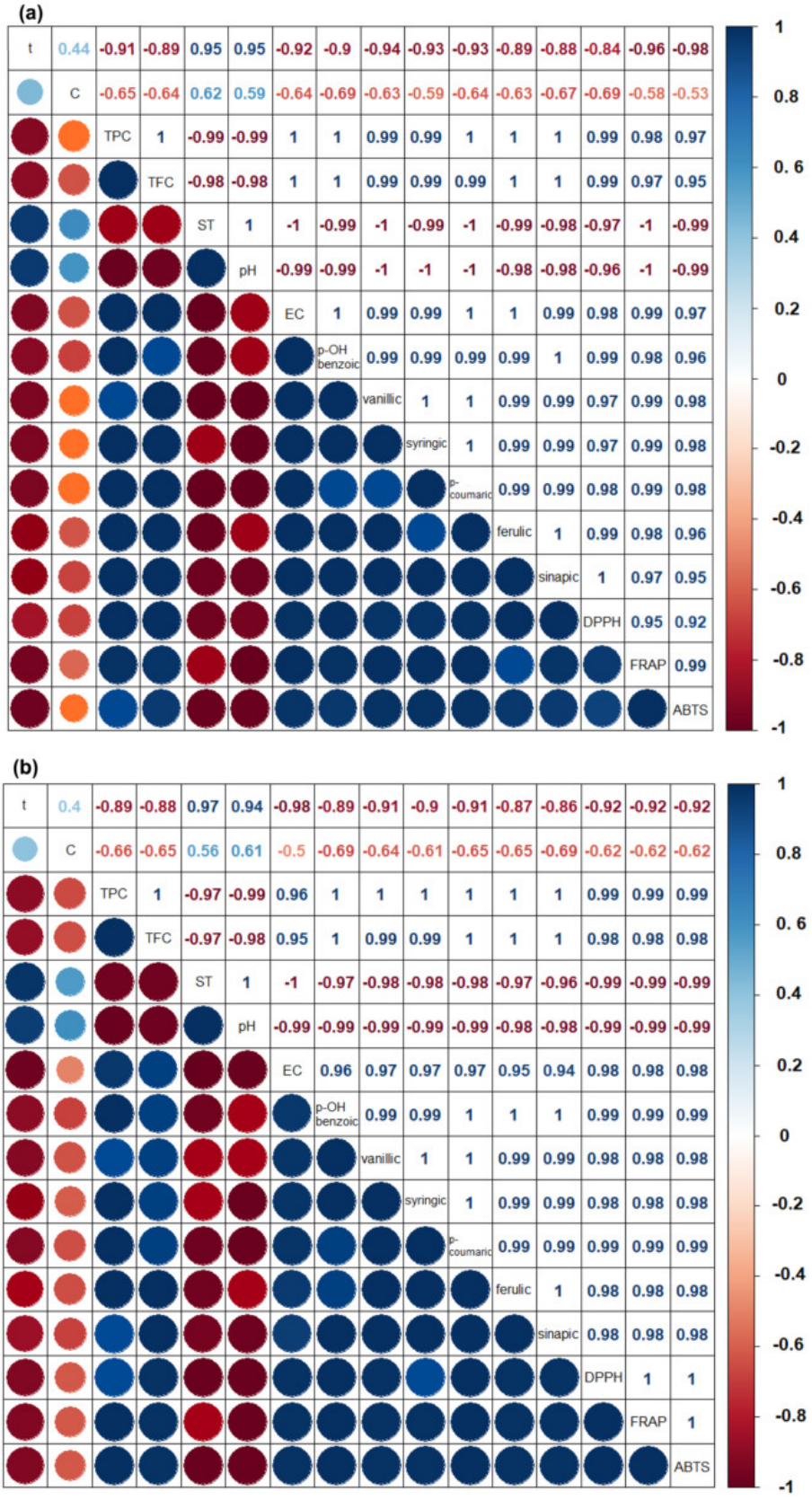


Figure 4. Correlations between TPC, TFC, phenolic acid content, antioxidant activities and extraction parameters; (**a**) UAE with ethanol, (**b**) MAE with ethanol. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circles are proportional to the correlation coefficients. Values of Spearman's rank correlation coefficient above 0.6 are described as a strong correlation; t—extraction time, C—solvent concentration.

and sinapic) provides a greater ability to donate H and subsequently stabilize radicals than the carboxylate group in hydroxybenzoic acids (p-OH benzoic, vanillic, syringic). Therefore, the type of phenolic acids and hence their chemical/molecular structures exhibit different antioxidant properties. Additionally, the presence of a carboxyl group in ferulic acid facilitates the anchoring of ferulic acid in the lipid bilayer, providing effective protection against lipid peroxidation[58]. Therefore, special attention should be paid to the recovery of phenolic acids from distillery stillage due to its high concentration of ferulic acid, which has a high antioxidant activity.

Economic aspects. Although distillery stillage extracts contain lower concentrations of phenolic compounds per gram of dry mass than raw plant materials, this waste is produced in billions of tons per year resulting in the extracted phenolic compounds up to tens of thousands of kilograms. Taking into account the economic aspects of using solvents to valorize the stillage, the production costs of solvents that were used in the present study are quite low because the boiling points of ethanol (78.4 °C) and methanol (64.7 °C) allow them to evaporate quickly, which shortens the processing time. Additionally, ethanol, which gave higher extraction yields than methanol in the present study, has been recognized as GRAS (Generally Recognized As Safe) solvent, hence its extracts can find applications in the production of functional foods, dietary supplements, animal feed, or cosmetics[59]. In the present study, the use of 80% solution of ethanol allowed the highest recovering of compounds with antioxidant activity, which, when compared with the use of pure ethanol, increased the economic viability of the obtained extracts. In preliminary studies (data not shown), the solid-to-solvent ratio was selected to be 1:30 (w/v), which is an optimal value considering not only extraction yield but also the cost of purification and concentration of extracts. When comparing the extraction methods, polyphenol recovery yield was about 1.3 times higher with 3-min MAE than with 5-min UAE. These extractions consumed 20.0 Wh (MAE) and 45.2 Wh (UAE) of energy. However, other studies reported that despite higher extraction yields in MAE than in UAE, UAE was suggested as a technique for a future scaling-up evaluation due to its simplicity and investment and operational costs[32]. Therefore, implementing these extraction techniques on an industrial scale will need the assessment of other factors determining the economic and environmental impact. To further increase the profitability of the examined method of distillery stillage valorization, anaerobic treatment of post-extracted stillage for biogas production should be considered.

Conclusions

The study suggests that distillery stillage can be considered a rich source of natural antioxidants for further use. Optimizing extraction procedure allowed high-value extracts to be obtained with the use of solvents available in any distillery. Phenolic acids were present mainly in the free form (92% of the total content), which allows to avoid pre-treatment or adding harmful reagents for hydrolysis before their recovery. The TFC, TPC, and phenolic acids content depended on the extraction conditions and the solvent type and concentration. With each extraction method, 80% ethanol yielded extracts that were the richest in polyphenols (up to 5.07 ± 0.03 mg GAE/g DM), which contributed to the observed antioxidant activity (26.73 ± 1.81 μmol TE/g DM). Due to providing 2.1 and 1.7 times more efficient recovery and 2.1 and 1.5 times higher antioxidant activity at lower solvent concentrations than CSLE, MAE and UAE can be considered "green" alternatives to CSLE and recommended for the recovery of polyphenols from distillery stillage.

Future studies on recovering polyphenols from distillery stillage should focus on the improvement of recovery efficiency (in terms of profitability, time, and energy consumption) to shorten the reaction time and decrease the amount of solvent used. The development of cheap and green extraction procedures, including searching for waste-derived solvents, that will allow for obtaining a valuable extract rich in bioactive compounds is still a challenging issue. In addition, further studies should focus on the methods of purification and concentration of recovered phenolic compounds to obtain products that will be applicable in the pharmaceutical, cosmetic, and food industries. To confirm the favorable applicability of these products for commercial applications, in vivo studies are needed.

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Author contributions

W.M.: Conceptualization, Methodology, Analysis, Investigation, Formal analysis, Visualization, Validation, Writing—original draft. M.Z.: Conceptualization, Methodology, Investigation, Supervision, Funding acquisition, Formal analysis, Project administration, Writing—original draft. K.B.: Formal analysis, Methodology, Investigation, Supervision, Writing—review & editing. I.W.: Formal analysis, Supervision, Writing—review & editing.

Competing interests

The authors declare no competing interests.

Additional information

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Recovery of polyphenols from distillery stillage by microwave-assisted, ultrasound-assisted and conventional solid-liquid extraction

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Supplementary Material

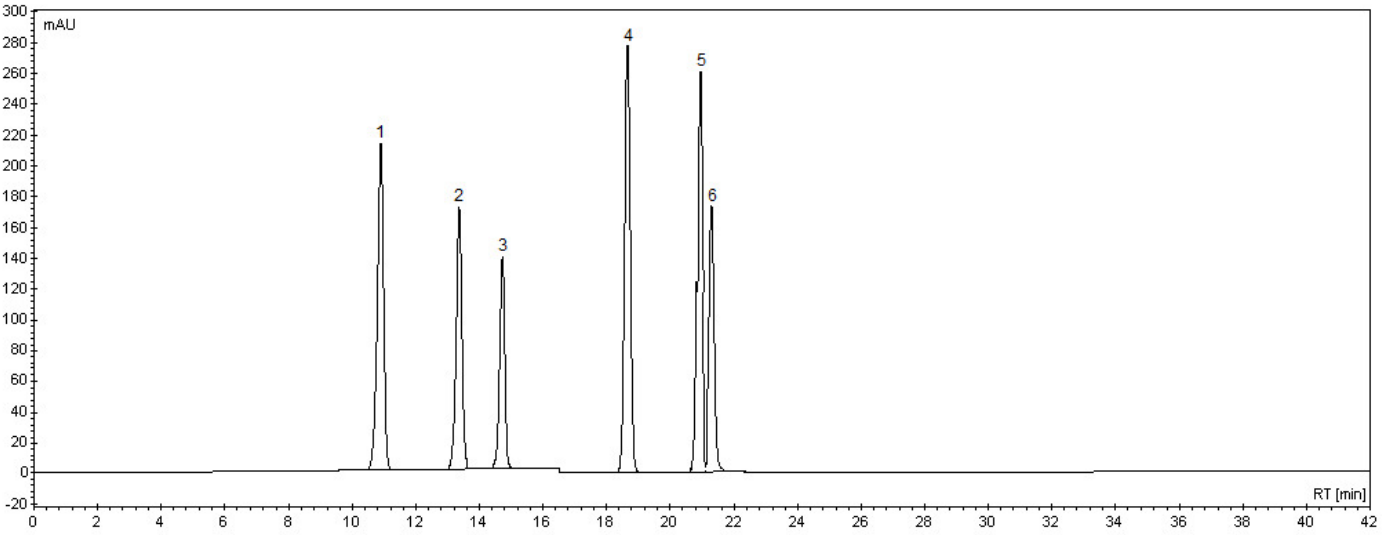
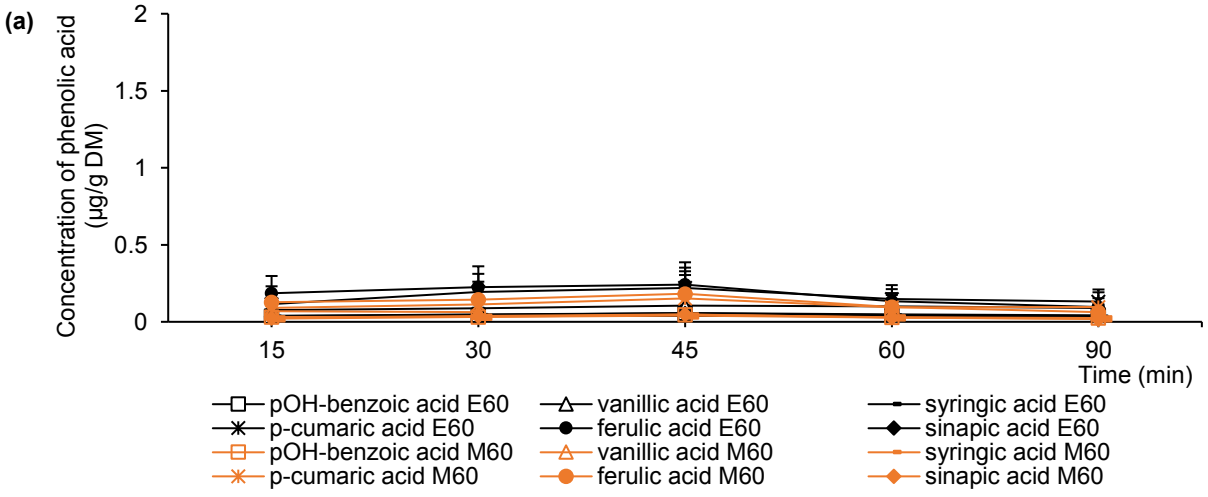
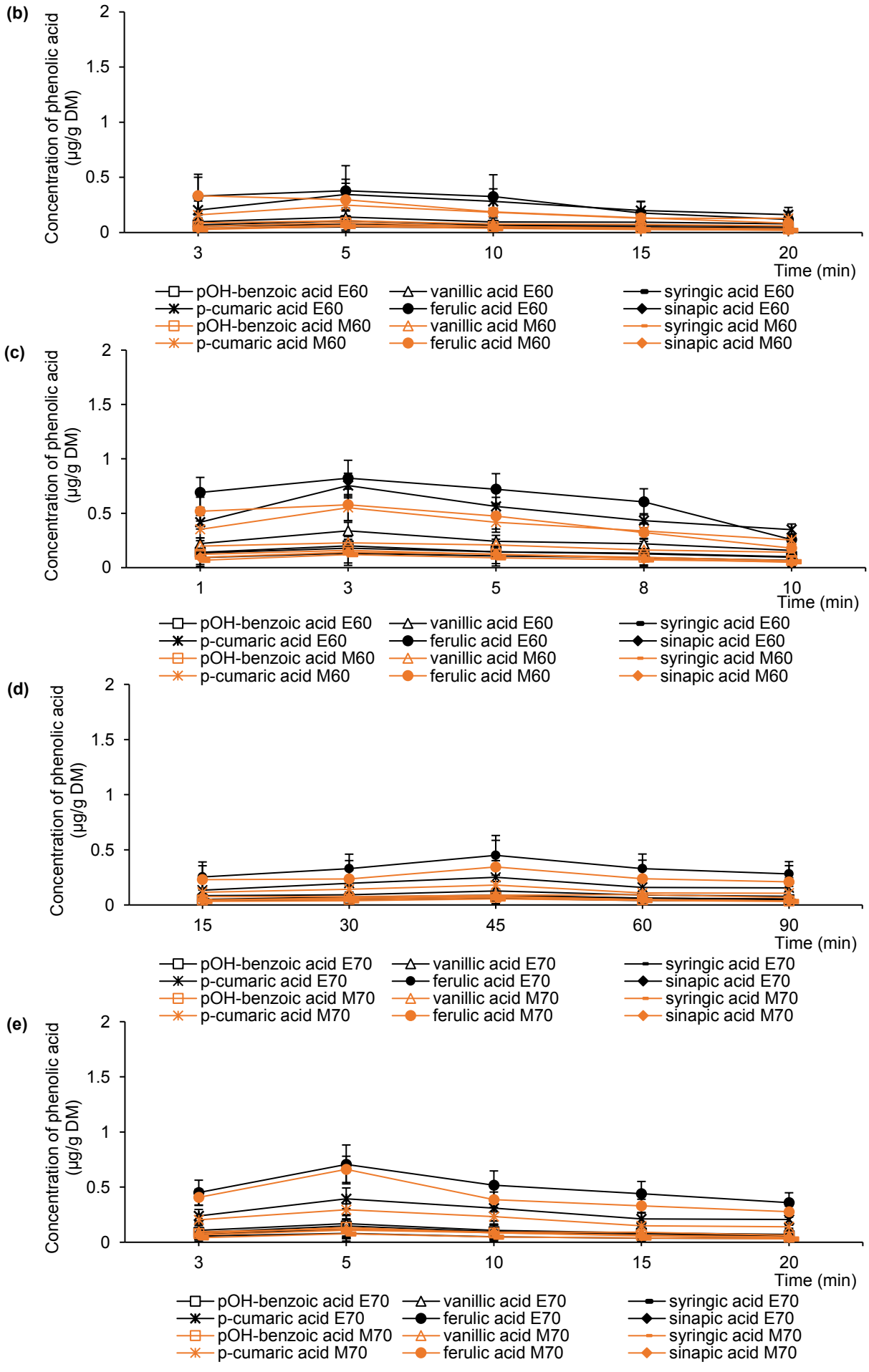
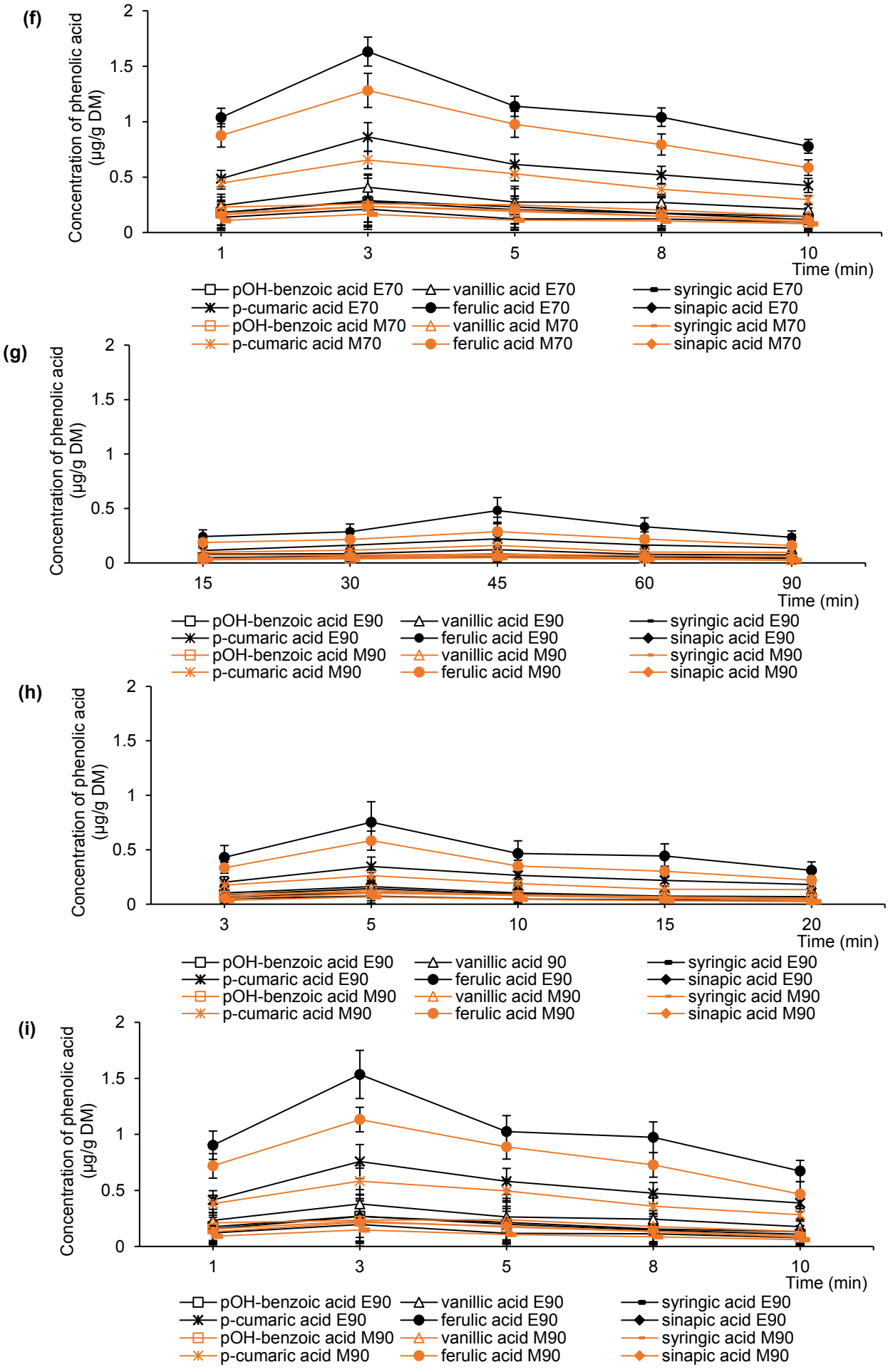


Figure 1SM. HPLC chromatogram of phenolic acid standards at 260 nm, peaks: 1 – p-OH benzoic acid, 2 – vanillic acid, 3 – syringic acid, and at 320 nm, peaks: 4 – p-coumaric acid, 5 – ferulic acid, 6 – sinapic acid




(b)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
3
5
10
15
20
Time (min)
pOH-benzoic acid E60
p-cumaric acid E60
pOH-benzoic acid M60
p-cumaric acid M60
vanillic acid E60
ferulic acid E60
vanillic acid M60
ferulic acid M60
syringic acid E60
sinapic acid E60
syringic acid M60
sinapic acid M60
(c)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
1
3
5
8
10
Time (min)
pOH-benzoic acid E60
p-cumaric acid E60
pOH-benzoic acid M60
p-cumaric acid M60
vanillic acid E60
ferulic acid E60
vanillic acid M60
ferulic acid M60
syringic acid E60
sinapic acid E60
syringic acid M60
sinapic acid M60
(d)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
15
30
45
60
90
Time (min)
pOH-benzoic acid E70
p-cumaric acid E70
pOH-benzoic acid M70
p-cumaric acid M70
vanillic acid E70
ferulic acid E70
vanillic acid M70
ferulic acid M70
syringic acid E70
sinapic acid E70
syringic acid M70
sinapic acid M70
(e)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
3
5
10
15
20
Time (min)
pOH-benzoic acid E70
p-cumaric acid E70
pOH-benzoic acid M70
p-cumaric acid M70
vanillic acid E70
ferulic acid E70
vanillic acid M70
ferulic acid M70
syringic acid E70
sinapic acid E70
syringic acid M70
sinapic acid M70


(f)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
1
3
5
8
10
Time (min)
pOH-benzoic acid E70
vanillic acid E70
syringic acid E70
p-cumaric acid E70
ferulic acid E70
sinapic acid E70
pOH-benzoic acid M70
vanillic acid M70
syringic acid M70
p-cumaric acid M70
ferulic acid M70
sinapic acid M70
(g)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
15
30
45
60
90
Time (min)
pOH-benzoic acid E90
vanillic acid E90
syringic acid E90
p-cumaric acid E90
ferulic acid E90
sinapic acid E90
pOH-benzoic acid M90
vanillic acid M90
syringic acid M90
p-cumaric acid M90
ferulic acid M90
sinapic acid M90
(h)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
3
5
10
15
20
Time (min)
pOH-benzoic acid E90
vanillic acid 90
syringic acid E90
p-cumaric acid E90
ferulic acid E90
sinapic acid E90
pOH-benzoic acid M90
vanillic acid M90
syringic acid M90
p-cumaric acid M90
ferulic acid M90
sinapic acid M90
(i)
Concentration of phenolic acid (µg/g DM)
2
1.5
1
0.5
0
1
3
5
8
10
Time (min)
pOH-benzoic acid E90
vanillic acid E90
syringic acid E90
p-cumaric acid E90
ferulic acid E90
sinapic acid E90
pOH-benzoic acid M90
vanillic acid M90
syringic acid M90
p-cumaric acid M90
ferulic acid M90
sinapic acid M90

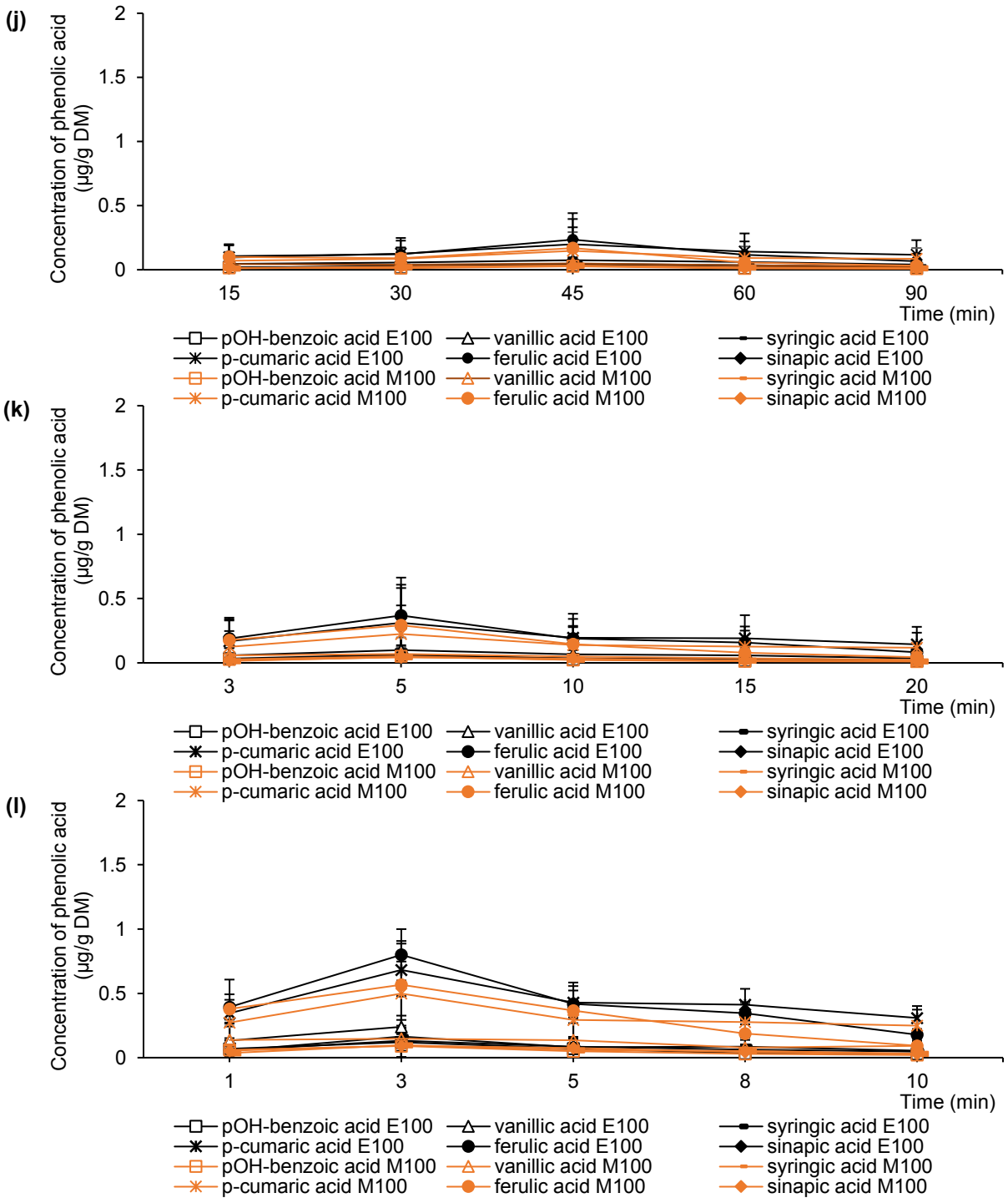


Figure 2SM. Concentrations of free phenolic acids in extracts obtained with **(a)** CSLE E60 and M60, **(b)** UAE E60 and M60, **(c)** MAE E60 and M60, **(d)** CSLE E70 and M70, **(e)** UAE E70 and M70, **(f)** MAE E70 and M70, **(g)** CSLE E90 and M90, **(h)** UAE E90 and M90, **(i)** MAE E90 and M90, **(j)** CSLE E100 and M100, **(k)** UAE E100 and M100, **(l)** MAE E100 and M100

Table 1SM. Efficiency of recovery of phenolic acids from distillery stillage using the standard addition method

Concentration of alcohol (%)	Type of extraction														
	CSLE15	CSLE30	CSLE45	CSLE60	CSLE90	UAE3	UAE5	UAE10	UAE15	UAE20	MAE1	MAE3	MAE5	MAE8	MAE10
E60	68.86	69.85	67.93	58.77	54.09	76.53	79.31	69.20	62.80	57.37	76.07	81.23	73.93	66.70	60.92
E70	73.99	77.03	70.56	61.91	54.96	80.98	85.58	75.69	68.52	61.73	84.01	89.03	78.76	70.65	63.92
E80	77.80	81.88	73.42	63.90	55.60	89.39	96.27	81.30	71.38	64.45	91.85	99.14	84.63	73.99	66.93
E90	70.74	73.92	69.67	60.23	54.50	78.92	82.80	71.89	65.36	59.60	81.00	85.91	75.23	68.51	62.80
E100	66.77	67.98	65.68	55.04	53.93	73.03	76.45	67.49	61.85	56.57	70.68	76.33	71.26	65.68	59.03
M60	59.49	67.52	57.69	54.19	52.85	71.83	75.28	64.52	61.05	55.35	72.63	77.46	72.38	66.02	59.91
M70	66.70	70.44	62.10	59.34	53.91	77.11	81.06	68.68	66.57	59.49	79.72	85.86	78.68	69.43	62.41
M80	69.77	72.59	66.21	60.64	54.56	81.86	87.22	73.52	69.91	61.04	84.65	90.28	81.35	72.79	64.44
M90	64.15	69.90	60.41	57.16	53.61	75.74	78.35	65.77	64.24	57.19	76.65	81.23	75.52	67.55	61.36
M100	57.06	56.72	55.34	52.45	52.05	67.48	71.57	62.54	59.69	54.79	68.48	73.68	65.87	65.16	58.53

Table 2SM. Antioxidant activities of distillery stillage extracts obtained with 60%, 70%, 90%, and 100% solvents

Type of extraction	Antioxidant Assays			Type of extraction	Antioxidant Assays			Type of extraction	Antioxidant Assays		
	ABTS (μmol TE/g DM)	DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)		ABTS (μmol TE/g DM)	DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)		ABTS (μmol TE/g DM)	DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)
CSLE15 E60	8.18±1.23	2.27±0.52	1.38±0.24	UAE3 E60	14.11±1.44	3.91±0.63	2.38±0.44	MAE1 E60	21.38±1.88	4.46±0.88	2.97±0.51
CSLE15 E70	8.38±1.12	2.40±0.48	1.52±0.27	UAE3 E70	14.45±1.46	4.13±0.69	2.61±0.46	MAE1 E70	23.51±1.92	4.90±0.96	3.27±0.68
CSLE15 E90	8.33±1.14	2.36±0.46	1.49±0.25	UAE3 E90	14.36±1.45	4.07±0.71	2.57±0.45	MAE1 E90	23.17±1.86	4.83±0.91	3.22±0.66
CSLE15 E100	8.09±1.19	2.18±0.39	1.29±0.21	UAE3 E100	13.95±1.41	3.76±0.60	2.23±0.41	MAE1 E100	20.10±1.41	4.19±0.74	2.79±0.41
CSLE15 M60	7.93±1.08	2.13±0.35	1.36±0.24	UAE3 M60	13.67±1.38	3.34±0.54	2.23±0.38	MAE1 M60	16.04±1.32	3.67±0.63	2.34±0.31
CSLE15 M70	8.12±1.15	2.27±0.38	1.47±0.27	UAE3 M70	13.99±1.42	3.56±0.56	2.38±0.43	MAE1 M70	17.11±1.48	3.92±0.62	2.58±0.38
CSLE15 M90	8.06±1.12	2.22±0.34	1.44±0.22	UAE3 M90	13.90±1.43	3.45±0.51	2.30±0.42	MAE1 M90	16.57±1.38	3.82±0.55	2.49±0.35
CSLE15 M100	7.85±1.14	2.06±0.31	1.28±0.25	UAE3 M100	13.55±1.36	3.23±0.48	2.16±0.36	MAE1 M100	15.50±1.33	3.55±0.59	2.21±0.29
CSLE30 E60	9.05±1.22	2.55±0.39	1.55±0.29	UAE5 E60	16.24±1.52	4.25±0.71	2.54±0.44	MAE3 E60	25.12±1.96	5.24±0.94	3.49±0.34
CSLE30 E70	9.30±1.35	2.70±0.42	1.82±0.31	UAE5 E70	16.49±1.58	4.50±0.76	2.89±0.48	MAE3 E70	26.20±2.03	5.46±0.97	3.64±0.39
CSLE30 E90	9.25±1.23	2.66±0.49	1.76±0.28	UAE5 E90	16.41±1.56	4.43±0.69	2.81±0.47	MAE3 E90	25.66±1.96	5.35±0.96	3.56±0.34
CSLE30 E100	8.96±1.09	2.45±0.45	1.49±0.19	UAE5 E100	16.10±1.52	4.09±0.52	2.33±0.45	MAE3 E100	24.59±1.84	5.13±0.89	3.42±0.36
CSLE30 M60	8.86±1.02	2.37±0.41	1.50±0.20	UAE5 M60	14.86±1.24	4.14±0.50	2.50±0.38	MAE3 M60	22.29±1.71	4.64±0.91	3.10±0.29
CSLE30 M70	9.02±1.12	2.56±0.51	1.68±0.18	UAE5 M70	15.41±1.39	4.27±0.53	2.68±0.47	MAE3 M70	24.92±1.53	5.19±0.74	3.46±0.38
CSLE30 M90	8.96±1.28	2.47±0.43	1.63±0.21	UAE5 M90	15.33±1.36	4.22±0.51	2.61±0.45	MAE3 M90	24.30±1.49	5.06±0.71	3.38±0.33
CSLE30 M100	8.31±1.19	2.30±0.33	1.48±0.16	UAE5 M100	14.59±1.25	3.98±0.47	2.29±0.40	MAE3 M100	21.98±1.21	4.58±0.63	3.05±0.27
CSLE45 E60	10.55±1.31	2.76±0.53	1.65±0.26	UAE10 E60	14.37±1.21	4.05±0.49	2.45±0.42	MAE5 E60	22.96±1.34	4.79±0.79	3.19±0.31
CSLE45 E70	10.72±1.36	2.93±0.55	1.88±0.30	UAE10 E70	14.76±1.29	4.27±0.54	2.88±0.49	MAE5 E70	26.98±1.96	5.62±0.89	3.75±0.48
CSLE45 E90	10.67±1.39	2.88±0.51	1.83±0.29	UAE10 E90	14.67±1.31	4.22±0.52	2.79±0.51	MAE5 E90	26.15±1.99	5.45±0.92	3.63±0.47
CSLE45 E100	10.47±1.41	2.66±0.39	1.51±0.27	UAE10 E100	14.22±1.18	3.89±0.38	2.39±0.38	MAE5 E100	22.40±1.64	4.66±0.77	3.11±0.28
CSLE45 M60	9.66±1.33	2.69±0.41	1.63±0.26	UAE10 M60	14.06±1.14	3.75±0.35	2.38±0.34	MAE5 M60	21.04±1.55	4.38±0.62	2.92±0.24
CSLE45 M70	10.02±1.37	2.77±0.46	1.74±0.29	UAE10 M70	14.32±1.23	4.06±0.41	2.66±0.41	MAE5 M70	22.77±1.64	4.75±0.78	3.16±0.34
CSLE45 M90	9.97±1.33	2.74±0.48	1.70±0.27	UAE10 M90	14.22±1.17	3.92±0.36	2.60±0.37	MAE5 M90	22.37±1.67	4.66±0.64	3.11±0.29
CSLE45 M100	9.48±1.29	2.59±0.34	1.49±0.23	UAE10 M100	13.96±1.13	3.65±0.37	2.35±0.24	MAE5 M100	19.85±1.29	4.14±0.59	2.76±0.22
CSLE60 E60	8.03±1.25	2.18±0.28	1.39±0.31	UAE15 E60	13.85±1.11	3.77±0.39	2.39±0.26	MAE8 E60	20.67±1.33	4.31±0.49	2.87±0.24
CSLE60 E70	8.20±1.41	2.31±0.24	1.55±0.25	UAE15 E70	14.14±1.27	3.98±0.36	2.67±0.39	MAE8 E70	23.05±1.46	4.80±0.52	3.20±0.58
CSLE60 E90	8.15±1.23	2.27±0.25	1.52±0.29	UAE15 E90	14.05±1.24	3.92±0.40	2.61±0.37	MAE8 E90	22.57±1.28	4.70±0.49	3.14±0.46
CSLE60 E100	7.93±1.26	2.10±0.21	1.30±0.27	UAE15 E100	13.67±1.13	3.62±0.33	2.24±0.26	MAE8 E100	19.39±1.14	4.04±0.35	2.70±0.28
CSLE60 M60	7.71±1.35	2.08±0.18	1.34±0.24	UAE15 M60	13.28±1.06	3.59±0.31	2.30±0.24	MAE8 M60	19.91±1.11	4.15±0.37	2.77±0.37
CSLE60 M70	7.93±1.16	2.18±0.22	1.45±0.27	UAE15 M70	13.67±1.24	3.77±0.35	2.50±0.28	MAE8 M70	21.62±1.37	4.50±0.46	3.00±0.36

CSLE60 M90	7.87±1.41	2.16±0.28	1.42±0.24	UAE15 M90	13.57±1.19	3.73±0.34	2.44±0.25	MAE8 M90	21.10±1.42	4.39±0.47	2.93±0.26
CSLE60 M100	7.61±1.34	2.00±0.23	1.28±0.29	UAE15 M100	13.12±1.02	3.45±0.29	2.20±0.21	MAE8 M100	19.01±1.14	3.96±0.34	2.64±0.37
CSLE90 E60	6.83±1.01	1.74±0.22	1.36±0.28	UAE20 E60	12.41±0.92	3.16±0.22	2.48±0.27	MAE10 E60	20.49±1.03	4.27±0.51	2.85±0.44
CSLE90 E70	6.96±1.11	1.86±0.27	1.39±0.22	UAE20 E70	12.66±0.98	3.39±0.24	2.53±0.29	MAE10 E70	20.95±1.07	4.37±0.56	2.91±0.36
CSLE90 E90	6.91±0.98	1.83±0.34	1.38±0.27	UAE20 E90	12.57±0.96	3.32±0.23	2.50±0.30	MAE10 E90	20.67±0.99	4.31±0.55	2.87±0.49
CSLE90 E100	6.75±0.94	1.67±0.35	1.23±0.19	UAE20 E100	12.28±0.89	2.98±0.19	2.20±0.20	MAE10 E100	18.22±0.88	3.80±0.64	2.53±0.21
CSLE90 M60	6.35±1.08	1.59±0.28	1.22±0.18	UAE20 M60	11.54±1.06	2.92±0.18	2.23±0.18	MAE10 M60	18.25±1.19	3.84±0.68	2.55±0.26
CSLE90 M70	6.50±1.07	1.75±0.24	1.25±0.17	UAE20 M70	11.82±1.04	3.18±0.33	2.28±0.23	MAE10 M70	18.90±0.96	3.94±0.75	2.62±0.33
CSLE90 M90	6.47±1.02	1.71±0.18	1.24±0.16	UAE20 M90	11.76±0.88	3.10±0.34	2.26±0.24	MAE10 M90	18.72±0.87	3.90±0.77	2.60±0.51
CSLE90 M100	6.24±1.12	1.55±0.15	1.21±0.17	UAE20 M100	11.35±0.93	2.81±0.26	2.15±0.19	MAE10 M100	17.76±0.83	3.70±0.46	2.47±0.55

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Attachment 3.

International Journal of
Environmental Research and Public Health

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Article

Processing of Distillery Stillage to Recover Phenolic Compounds with Ultrasound-Assisted and Microwave-Assisted Extractions

Wioleta Mikucka [1,*], Magdalena Zielinska [1], Katarzyna Bulkowska [1] and Izabela Witonska [2]

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Abstract: This study investigated the effect of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) conditions (extraction time, acetone concentration, solid-to-solvent ratio) on the efficiency of polyphenol recovery from distillery stillage and antioxidant activity of the extracts. The highest total polyphenol content, flavonoid content, and phenolic acid content were obtained with 10-min UAE and 5-min MAE at a solid-to-acetone ratio of 1:15 (*w*:*v*). Recovery yield was the highest with an aqueous solution of 60% acetone, confirming the results of Hansen Solubility Parameter analysis. Although UAE resulted in approximately 1.2 times higher extraction yield, MAE showed a better balance between extraction yield and energy consumption exhibited by its 3-fold higher extraction rate than that of UAE. Content of total polyphenols and phenolic acids strongly correlated with antioxidant activity, indicating that these compounds provide a substantial contribution to the bioactive properties of the extracts. Six phenolic acids were extracted, predominately ferulic and p-coumaric acids, and free forms of these acids constituted 91% of their total content, which opens various possibilities for their application in the food, cosmetics, and pharmaceutical industries.

Keywords: distillery by-product; bioactive compounds; phenolic acids; flavonoids; HPLC; waste valorization

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Citation: Mikucka, W.; Zielinska, M.; Bulkowska, K.; Witonska, I. Processing of Distillery Stillage to Recover Phenolic Compounds with Ultrasound-Assisted and Microwave-Assisted Extractions. *Int. J. Environ. Res. Public Health* **2022**, *19*, 2709. https://doi.org/10.3390/ijerph19052709

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1. Introduction

Sustainable industrial development and waste valorization have become key industry concerns. Distilleries are one of the most polluting industries since 88% of their raw materials are converted into distillery stillage, which is difficult to handle due to its low pH, high organic matter content, and dark brown color [1]. The utilization of the by-products from alcohol production is based on using them as animal feed [2], field fertilizer [1], and for biogas production [3]. However, such uses of stillage still fail to completely solve the problem of its management. Therefore, there is an emphasis on the circular economy concept in which the by-products remain in circulation for as long as possible. The employment of such an approach resulted in the development of processes that first ensure the recovery of valuable chemical compounds for the economy, and then valorization of the waste. Among the methods of stillage utilization, recovery of bioactive compounds represents a novel approach, which would not only reduce the pollution generated during alcohol production but also contribute to the economic competitiveness and sustainable development of the distillery industry.

Bioactive compounds present in plant-origin materials, such as waste from the distillery industry, include polyphenols [4]. Polyphenols are classified according to their structure as phenolic acids, flavonoids, and tannins. These molecules have an aromatic ring containing one or more hydroxyl groups; they function as hydrogen donors, antioxidants, and oxygen quenchers [5]. These functions render polyphenolic substances of great interest,

owing to their role in preventing various human diseases [4,6]. Due to the biological properties, polyphenols have the potential to be used in food, pharmaceutical, cosmetics, and organic fine chemistry industries. These compounds find applications related to food dying, food preservation, bioactive packaging, improvement of the physicochemical properties of starch, and production of prebiotic ingredients, surfactants, cosmetic products, hydrogels and nanocomplexes [7]. Phenolic acids are among the most antioxidant metabolites of cereal crops [8]. The antioxidant properties of phenolic compounds are related to their ability to scavenge free radicals, disrupt radical chain reactions, and chelation of metals. Due to a variety of simultaneous reactions between antioxidants and free radicals, assays based on the mechanism of hydrogen atom transfer (HAT) and single electron transfer (SET) are used to determine antioxidant activity [9]. Due to the simplicity and low cost of the analysis, the ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay (based on the HAT mechanism) is employed more often than the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and FRAP (ferric reducing antioxidant power) assay (both based on the SET mechanism). In addition, the ABTS assay is of particular interest regarding its use for measuring the activity in plant extracts as the absorbance of the wavelength at 734 nm, which is applied for this measurement, eliminates color interference [10]. However, these three tests have shown different results for individual crop species. Yu et al. [11] displayed correlations of antioxidant activity with FRAP and DPPH tests used for wheat samples, whereas Awika et al. [9] observed a high correlation between ABTS, DPPH, and FRAP results among sorghum and its products. Therefore, to show a relationship between the structure of polyphenols from a newly tested waste material and their antioxidant activity, it is advisable to conduct several types of assays to determine the antioxidant activity of the recovered bioactive compounds.

The efficiency of polyphenol recovery depends on the solvent type and the extraction method, which should not chemically modify the extracted compounds. Typically, water or aqueous mixtures of ethanol and methanol in addition to acetone are used to extract antioxidant compounds [12]. Acetone is a slightly toxic solvent that is widely employed in the production of drugs, cosmetics, and in biomedicine. Khan et al. [13] showed that acetone extracts from the *Salvia moorcroftiana* plant exhibit antifungal and antibacterial activity against animal and plant pathogens. Sasmal et al. [14] determined the antipyretic effect of acetone extract from seeds of *Saraca asoca* (Roxb.) in rats. However, to maximize the polyphenol yield, the process parameters should be optimized for the extraction of these compounds from particular waste materials.

The efficiency of extraction of bioactive compounds from biomass can be improved by using physical cell-disruption techniques, such as ultrasound-assisted extraction (UAE) or microwave-assisted extraction (MAE) [4]. For example, optimized MAE can yield up to five times more ferulic acid than conventional solid–liquid extraction techniques [15]. UAE is very efficient, providing, in only an hour, extraction yields more than 6–35% higher than those of conventional extraction methods that often take up to 12 h [12]. Both MAE, based on rapid heating of the solvent by electromagnetic energy (causing molecular movement through ionic conductivity and dipole rotation), and UAE, based on acoustic cavitation, increase the penetration of the solvent into the substrate, improving the mass transfer rate. MAE and UAE offer the following advantages over conventional methods: they can be operated in the absence of light and oxygen; they reduce the consumption of organic solvents; and they can shorten the extraction time [4]. In general, the higher the dielectric constant or the more polar the solvent, the greater its ability to absorb microwaves, thus improving the mass transfer rate, which increases the solvation of the extracted compounds. However, this does not guarantee better extraction results, especially in the case of thermolabile compounds such as polyphenols [15]. Thus, due to the different chemical properties of compounds, it is necessary to optimize MAE and UAE conditions for every solvent and every type of extracted compound. Additionally, the content of polyphenols varies in different types of biomass [16]. For example, Nayak et al. [17] compared MAE and UAE with 51% acetone for the recovery of polyphenols from *Citrus sinensis* peels. They recovered polyphenols of 12.09 mg gallic acid equivalent (GAE)/g with the activity of 337.162 mg

Trolox equivalent (TE)/g with MAE and 10.35 mg GAE/g and with 433.084 mg TE/g with UAE. Cherif et al. [18] recovered 94.62 mg ferulic acid equivalent/g from wheat waste with UAE using deep eutectic solvents. The extraction of phenolic compounds from banana peels with MAE using boiling deionized water as a solvent resulted in the content of the phenolic compounds, FRAP, and DPPH antioxidant properties of 53.76 mg GAE/g, 95.52 mg TE/g, and 95.29 mg TE/g, respectively [19]. Thus, because of these very divergent results, there is a need to optimize the conditions of MAE- and UAE-assisted extraction of polyphenols from distillery stillage with a particular solvent.

Several studies reported that the extraction methods and properties of the recovered compounds strongly depend on their structural features and the composition of the matrix, the type of compounds, and the strength of their association with the matrix [16]. Due to their bifunctional nature, phenolic acids can form both ester and glycosidic bonds through reactions involving their carboxyl and hydroxyl groups, respectively, which allow these compounds to form bonds with the cell wall [20]. Phenolic compounds also bind with sugars, fatty acids, and proteins. However, in an acidic environment, esters and glycosidic bonds may be hydrolyzed, and microwaves and ultrasounds may affect the release of phenolic acids from the matrix, increasing the amount of free phenolic acids. Although the hydrolysis conditions significantly affect the overall yield and profile of the phenolic acids, p-coumaric, caffeic, and ferulic acids are degraded under hot acidic conditions, which may reduce the antioxidant activity of the extract [21]. Therefore, to maximize the polyphenol extraction yield and to completely determine the quantity and quality of phenolic acids that maintain their antioxidant activity, it is important to optimize hydrolysis conditions and to consider bound phenolic compounds as most of the phenolic compounds in plant materials are present in this form [21].

The optimization of polyphenol recovery from distillery stillage with UAE and MAE and determination of the antioxidant activity of the extracts represents a novel approach to the utilization of cereal processing by-products generated in alcohol production. Therefore, the main objectives of this study were to investigate polyphenol recovery from distillery stillage with the use of acetone as a solvent to determine how UAE and MAE conditions (extraction time, solvent concentration, and solid-to-solvent ratio) affect (i) the yield of total polyphenols, (ii) the content of flavonoids in the extracts, (iii) the type and content of phenolic acids in the extracts, (iv) kinetic parameters of extraction, and (v) the antioxidant activity of the extracts.

2. Materials and Methods

2.1. Sample Preparation

Distillery stillage was collected after the production of concentrated crude ethyl alcohol from cereals, mainly wheat and rye, in a distillery located in northeast Poland. The distillery stillage samples contained the following characteristics: 79.9 $\pm$ 3.0 g Total Solids/kg fresh weight, 76.2 $\pm$ 2.4 g Volatile Solids/kg fresh weight, 47,000 $\pm$ 5300 mg COD/L, 4345 $\pm$ 5 mg N_{tot}/L, 280 $\pm$ 2 mg P_{tot}/L, 789 $\pm$ 3 mg CH_3COOH/L, alkalinity of 182.8 $\pm$ 1.4 meq/L, and a pH of 4.52. After transportation to the laboratory, the stillage was frozen and then freeze-dried (STERIS Lyovac GT2 freeze dryer with a Leybold Trivac vacuum pump, Hamburg, Germany). The freeze-dried samples were stored in tightly closed containers until extractions were performed.

2.2. Extraction Procedure

For polyphenol extraction, 1 g of the freeze-dried sample of distillery stillage was taken to each extraction vessel. UAE and MAE were performed with aqueous solutions of acetone at concentrations of 40%, 60%, 80%, and 100%. For the extraction, freeze-dried samples of the stillage were mixed with acetone at ratios of 1:15 (*w*:*v*) and 1:30 (*w*:*v*). UAE was carried out at a frequency of 25 kHz in an ultrasonic bath (InterSonic IS-5.5, Olsztyn, Poland) in a continuous mode. Water remained circulating in the bath to maintain a stable temperature and to avoid overheating the sample as an effect of cavitation. The extraction

times were 3, 5, 10, 15, and 20 min. MAE was conducted in a microwave oven (MARSXpress 240/50, CEM, Matthews, NC, USA) in TFM extraction vessels for 1, 3, 5, 8, and 10 min at 400 W and 50 °C. The UAE and MAE extracts were centrifuged (Centrifuge MPW-380, Warszawa, Poland) for 10 min at 10,000 rpm. In the supernatants (final extracts) pH (pH meter, HANNA Instruments HI 221, Cluji-Napoca, Romania), and surface tension (ST) (K100C tensiometer, Hamburg, Germany) were measured at room temperature.

2.3. Determination of Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC)

TPC was measured according to the method described by Singletion et al. [22] with modifications. Briefly, 0.25 mL of each extract was mixed with 0.25 mL of Folin–Ciocalteu reagent (F-C) (Sigma-Aldrich, Saint Louis, MO, USA) and 0.5 mL of 14% Na_2CO_3. Then, the mixture was incubated for 30 min in the dark at room temperature. TFC was determined according to Quettier-Deleu et al. [23] with modifications. Briefly, 0.5 mL of the extract was mixed with 0.5 mL of 2% $AlCl_3$ and 1 mL of 5% $NaNO_2$ and incubated for 40 min in the dark at room temperature. TPC and TFC were measured with a UV-Vis spectrophotometer (Varian Cary 50 Scan UV-Visible, Belrose, Australia) at 760 nm and 415 nm, respectively. TPC was expressed as milligrams of gallic acid equivalent per gram dry mass (mg GAE/g DM) and TFC as milligrams of quercetin equivalent per gram dry mass (mg QUE/g DM). Gallic acid and quercetin for curve calibration was sourced from Sigma-Aldrich (Saint Louis, MO, USA).

2.4. Determination of Phenolic Acid Content

The collected final extracts from UAE and MAE were evaporated to dryness (Vacuum Rotavapor R-210, BUCHI, Flawil, Switzerland) at 50 °C. Then, 20 mL of distilled water acidified to pH 2 with 6M HCl was added to the dry extracts to dissolve the precipitate formed by evaporating the solvent. Free phenolic acids were extracted from the aqueous solution four times using diethyl ether. The diethyl ether extracts were dried on a rotary vacuum evaporator and redissolved in 1 mL of methanol. The samples thus prepared were used for chromatographic analysis (HPLC).

For recovery of bound phenolic acids, hydrolysis was performed. Distillery stillage (1 g) was mixed with 20 mL of 2M NaOH for 4 h. Then, the mixture was acidified to pH 2 with 6M HCl and centrifuged (Centrifuge MPW-380, Warszawa, Poland) for 10 min at 10,000 rpm. The supernatants were extracted four times with 20 mL of diethyl ether. The mixture of phenolic acids and diethyl ether was evaporated to dryness using a rotary vacuum evaporator. The dry solid was dissolved in 1 mL of methanol (purity: 99.9%, Sigma-Aldrich, Saint Louis, MO, USA) and then the phenolic acids were identified by HPLC.

Free and bound phenolic acids were identified chromatographically, according to the method of Chiremba et al. [24] with modifications. The compounds were separated by an HPLC device (Varian, Belrose, Australia) equipped with a UV-Vis detector (Varian ProStar 325, Belrose, Australia) and a Supelcosil C18 column (150 mm × 4.6 mm, 5 µm) (Sigma-Aldrich, Saint Louis, MO, USA). The mobile phase of the elution comprised acetonitrile and formic acid at a ratio of 99.85:0.15 (*v*:*v*) (eluent A) and water and formic acid at a ratio of 99.85:0.15 (*v*:*v*) (eluent B). The purity of the acetonitrile measured 99.9%, formic acid was 98–100% and water PLUS was sustainable for HPLC (Sigma-Aldrich, Saint Louis, MO, USA). The separation time was 42 min at a flow rate of 1 mL/min and a temperature of 35 °C. The analysis used the following gradient elution: 0–18 min, 1–96% B; 18–35 min, 96–82% B; 35–40 min, 82–75% B. P-OH benzoic, vanillic, and syringic acids were detected at 260 nm; p-coumaric, ferulic and sinapic acids, at 320 nm. Identification of compounds was achieved by comparison to the retention time and UV spectra of reference phenolic acids (Sigma-Aldrich, Saint Louis, MO, USA). The purity of the standards measured 95–99%. Due to the lack of reports on the concentration of phenolic acids in by-products from alcohol production from cereals, a wide range of standard solutions was used to render the calibration curves.

2.5. Antioxidant Activity

The antioxidant activity of polyphenols in the extracts was measured with ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidants power) assays.

2.5.1. ABTS Radical Scavenging Assay

The ABTS free radical scavenging assay was performed as described by Re et al. [25] with some modifications. Briefly, 10 mL of ABTS (Sigma-Aldrich, Saint Louis, MO, USA) was mixed with 0.1 mL $K_2S_2O_8$ and incubated in the dark at room temperature for 16 h. Then, 3 mL of the solution was mixed with 40 µL of the extract and incubated in the dark at room temperature for 6 min. The absorbance at 734 nm was measured (Varian Cary 50 Scan UV-Visible Spectrophotometer, Belrose, Australia). The results were expressed as micromoles of Trolox equivalent per gram dry mass (µmol TE/g DM). Trolox was sourced from Sigma-Aldrich (Saint Louis, MO, USA).

2.5.2. DPPH Radical Scavenging Assay

The DPPH free radical scavenging assay was performed according to Moure et al. [26]. Briefly, 2 mL of DPPH solution (Sigma-Aldrich, Saint Louis, MO, USA) was mixed with 0.2 mL of extract, then the mixture was incubated in the dark at room temperature for 16 min. The absorbance at 515 nm was measured (Varian Cary 50 Scan UV-Visible Spectrophotometer, Belrose, Australia). The results were expressed as micromoles of Trolox equivalent per gram dry mass (µmol TE/g DM).

2.5.3. FRAP Assay

The reducing capacity of the extracts was determined by the FRAP assay, according to Benzie and Strain [27]. FRAP reagent (Sigma-Aldrich, Saint Louis, MO, USA) and extract were incubated for 10 min at 37 °C. The absorbance at 593 nm was measured (Varian Cary 50 Scan UV-Visible Spectrophotometer, Belrose, Australia). The ability of the sample to reduce iron ions was calculated based on a calibration curve made by preparing an $FeSO_4$ solution. The results were expressed as micromoles of $FeSO_4$ equivalent per gram dry mass (µmol $FeSO_4$/g DM).

2.6. Calculations and Statistical Analysis

All extractions were performed in duplicate, and analyses were performed in triplicate. Spearman's rank correlation coefficient (r_s) was used to quantify the relationships between polyphenol content, antioxidant activities, and extraction parameters of UAE and MAE. The correlation matrix was visualized using a correlogram with RStudio Version 1.2.1335 using the "corrplot" package. For the statistical analysis, STATISTICA 13.1 (StatSoft) was used, and $p \leq 0.05$ was defined as significant.

To describe the effect of operating parameters on the extraction kinetics, second-order kinetics was used [28], which can be expressed as in Equations (1) and (2):

$$C_t = \frac{k \cdot t \cdot C_e^2}{1 + k \cdot t \cdot C_e} \quad (1)$$

$$r = k \cdot C_e^2 \quad (2)$$

where: C_t–concentration of phenolic acids (µg/g DM) in the extract at an extraction time t (min), k–the rate constant for extraction (g DM/(µg·min)), C_e–equilibrium concentration of the compound (µg/g DM) in the extract, r–the rate of extraction (µg/(g DM·min)).

3. Results and Discussion

3.1. Polyphenol Extraction Assisted by Microwaves and Ultrasounds

Extraction of bioactive compounds from waste is a complex process involving various steps such as penetration of solvent molecules, the release of matrix-related compounds,

and the dissolution of compounds in the solvent that is based on their affinity to the solvents. Therefore, in this study, optimization of recovery of polyphenols from distillery stillage included investigation of the solid-to-solvent ratio, the concentration of solvent, and the duration of MAE and UAE.

The authors' previous study on the use of ethanol and methanol for extracting polyphenols from distillery stillage provided the observation that UAE and MAE produced about 2-times higher polyphenol yield with over 9-times shorter extraction times than conventional solid–liquid extraction. Therefore, in this study, the authors focused on the UAE and MAE of polyphenols from distillery stillage. When using acetone as a solvent, the compound yield extracted by UAE was close to that of MAE, but higher TPC and TFC values were still obtained from UAE than from MAE. The TPC in the extracts ranged from 1.15 ± 0.23 mg GAE/g DM to 3.83 ± 0.61 mg GAE/g DM with UAE (Figure 1a) and from 1.00 ± 0.12 mg GAE/g DM to 3.26 ± 0.45 mg GAE/g DM with MAE (Figure 1b). The TFC values ranged from 0.24 ± 0.18 mg QUE/g DM to 0.72 ± 0.58 mg QUE/g DM with UAE and from 0.21 ± 0.09 mg QUE/g DM to 0.61 ± 0.17 mg QUE/g DM with MAE (Table 1). The microwave energy and acoustic cavitation represent the main factors that affect the efficiency of phenolic compound recovery in MAE and UAE, respectively. During UAE, acoustic cavitation causes cavitation bubbles to accummulate at sufficiently high pressures and then to collapse or implode, releasing large amounts of energy. This simultaneously causes high shear forces and turbulence around the cavitation bubbles. Electromagnetic energy in MAE also disturbs the structure of the distillery stillage particles, which leads to the release of compounds from the material matrix. However, excessively high microwave energy in MAE and over-pressurization from ultrasound cavitation in UAE may contribute to overheating the sample. Although an increase in temperature may result in an increase in solubility, diffusion rate and mass transfer of polyphenols, due to a decrease in viscosity of extraction solvent, polyphenols are sensitive to high temperatures [29]. To avoid degradation of these bioactive compounds in the present study, UAE was conducted at room temperature and MAE was conducted at 50 °C. On the other hand, the recovery of phenolic compounds from plant materials that are more closely bound to the cell wall [17,19,29], requires higher inputs of energy, temperature, and pressure for their release. Therefore, the first step in the recovery of phenolic compounds is to identify the most effective MAE and UAE conditions. In our preliminary study, the most effective level of the microwave was identified as 400 W, and the most effective amplitude of ultrasound treatment was found to be 100%. Compared to conventional heating processes, microwave heating involves a rapid increase in volume temperature without surface overheating, better process control, and higher energy efficiency, with fewer adverse effects on antioxidant activity and bioactive content [17]. Additionally, microwaves induce translational and rotational molecular movement by changing the structure of enzymes without directly breaking the covalent bonds between the polar groups in the enzyme [30]. Marszałek et al. [31] reported that polyphenol oxidase activity in strawberries was reduced by 80% during microwave heating (between 300 and 900 W). Therefore, microwaves may inhibit the enzymatic degradation of polyphenols and ensure their higher stability.

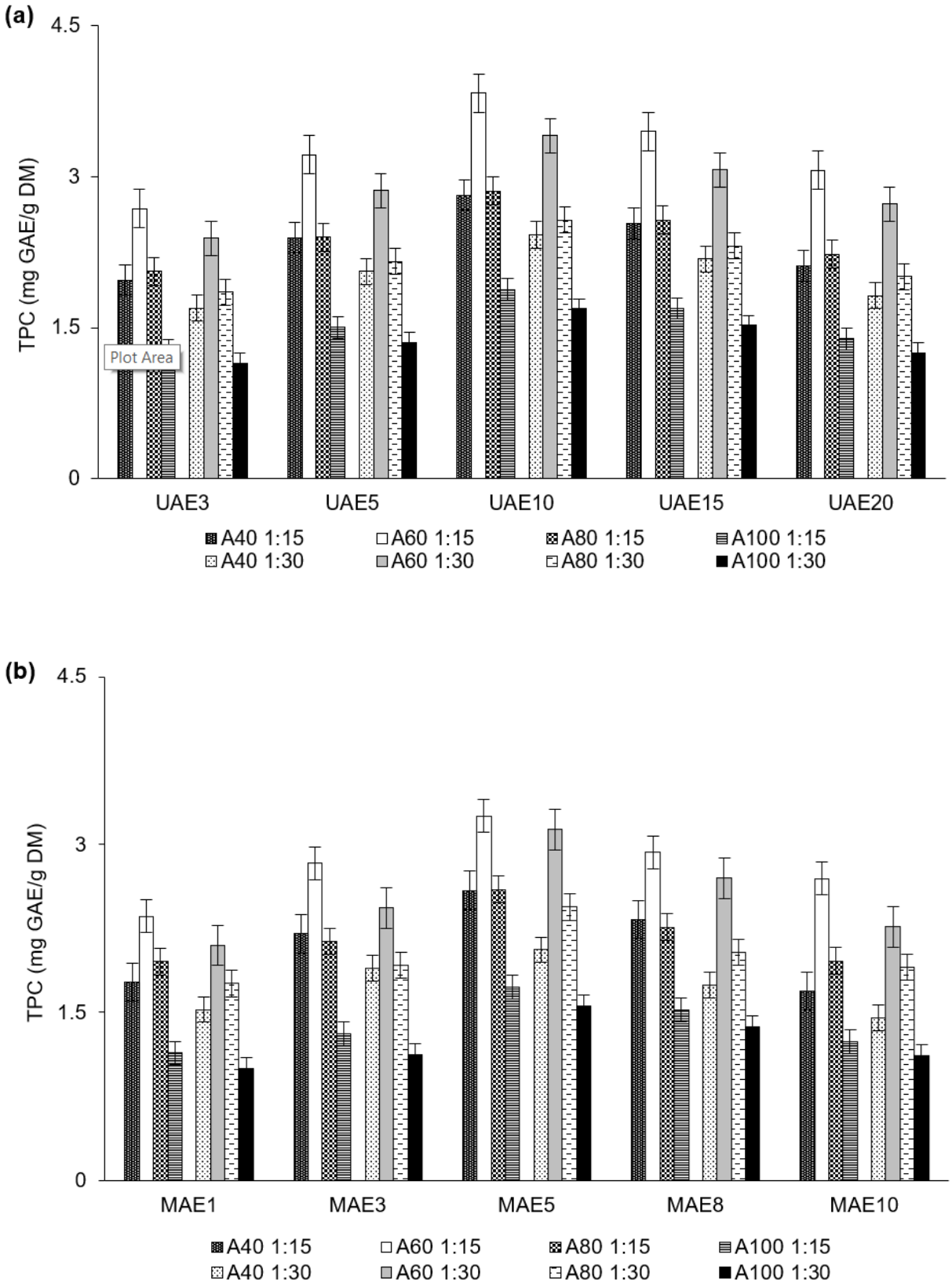


Figure 1. TPC that was extracted from distillery stillage with UAE (**a**) and MAE (**b**). In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time; the values after A (acetone) show the solvent concentration; and finally, the solid-to-solvent ratio is given.

Table 1. TFC that was extracted from distillery stillage with UAE and MAE. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time; the values after A (acetone) show the solvent concentration.

	Extraction	TFC (mg QUE/g DM)	Extraction	TFC (mg QUE/g DM)
Solid-to-solvent ratio of 1:15 (*w*:*v*)	UAE3 A40	0.40 ± 0.10	MAE1 A40	0.36 ± 0.44
	UAE3 A60	0.50 ± 0.18	MAE1 A60	0.44 ± 0.36
	UAE3 A80	0.43 ± 0.09	MAE1 A80	0.40 ± 0.38
	UAE3 A100	0.26 ± 0.16	MAE1 A100	0.23 ± 0.27
	UAE5 A40	0.49 ± 0.24	MAE3 A40	0.45 ± 0.31
	UAE5 A60	0.60 ± 0.42	MAE3 A60	0.53 ± 0.56
	UAE5 A80	0.50 ± 0.36	MAE3 A80	0.44 ± 0.41
	UAE5 A100	0.31 ± 0.38	MAE3 A100	0.27 ± 0.22
	UAE10 A40	0.58 ± 0.39	MAE5 A40	0.52 ± 0.58
	UAE10 A60	0.72 ± 0.58	MAE5 A60	0.61 ± 0.17
	UAE10 A80	0.59 ± 0.42	MAE5 A80	0.54 ± 0.55
	UAE10 A100	0.38 ± 0.37	MAE5 A100	0.35 ± 0.43
	UAE15 A40	0.52 ± 0.26	MAE8 A40	0.47 ± 0.33
	UAE15 A60	0.64 ± 0.54	MAE8 A60	0.57 ± 0.41
	UAE15 A80	0.53 ± 0.55	MAE8 A80	0.47 ± 0.22
	UAE15 A100	0.35 ± 0.49	MAE8 A100	0.32 ± 0.18
	UAE20 A40	0.43 ± 0.57	MAE10 A40	0.35 ± 0.19
	UAE20 A60	0.57 ± 0.61	MAE10 A60	0.52 ± 0.59
	UAE20 A80	0.46 ± 0.55	MAE10 A80	0.41 ± 0.27
	UAE20 A100	0.28 ± 0.42	MAE10 A100	0.25 ± 0.12
Solid-to-solvent ratio of 1:30 (*w*:*v*)	UAE3 A40	0.35 ± 0.21	MAE1 A40	0.31 ± 0.16
	UAE3 A60	0.49 ± 0.26	MAE1 A60	0.43 ± 0.38
	UAE3 A80	0.38 ± 0.32	MAE1 A80	0.36 ± 0.41
	UAE3 A100	0.24 ± 0.18	MAE1 A100	0.21 ± 0.09
	UAE5 A40	0.42 ± 0.23	MAE3 A40	0.38 ± 0.27
	UAE5 A60	0.58 ± 0.44	MAE3 A60	0.50 ± 0.42
	UAE5 A80	0.45 ± 0.37	MAE3 A80	0.40 ± 0.39
	UAE5 A100	0.28 ± 0.19	MAE3 A100	0.23 ± 0.27
	UAE10 A40	0.50 ± 0.43	MAE5 A40	0.42 ± 0.34
	UAE10 A60	0.69 ± 0.54	MAE5 A60	0.64 ± 0.59
	UAE10 A80	0.53 ± 0.51	MAE5 A80	0.50 ± 0.39
	UAE10 A100	0.35 ± 0.38	MAE5 A100	0.32 ± 0.26
	UAE15 A40	0.45 ± 0.52	MAE8 A40	0.36 ± 0.14
	UAE15 A60	0.62 ± 0.59	MAE8 A60	0.55 ± 0.48
	UAE15 A80	0.48 ± 0.58	MAE8 A80	0.42 ± 0.44
	UAE15 A100	0.32 ± 0.48	MAE8 A100	0.29 ± 0.22
	UAE20 A40	0.37 ± 0.36	MAE10 A40	0.30 ± 0.19
	UAE20 A60	0.56 ± 0.25	MAE10 A60	0.49 ± 0.34
	UAE20 A80	0.41 ± 0.58	MAE10 A80	0.39 ± 0.26
	UAE20 A100	0.26 ± 0.42	MAE10 A100	0.23 ± 0.29

3.1.1. Effect of Extraction Time on Polyphenol Recovery

The duration of extraction plays a key role in the recovery of phenolic compounds as it affects the stability of the bioactive compounds since UAE and MAE generate heat, and, in addition, obtaining a high yield over a shorter time period is much more productive. When the time of UAE was increased from 3 to 10 min, the amount of phenolic compounds that were extracted increased by about 43%, but further extension of this time decreased the recovery (Figure 1a, Table 1). At the longest duration of 20 min, the content of extracted compounds was similar to that obtained at 3 min. Regarding MAE, increasing the extraction time from 1 to 5 min increased the content of recovered polyphenols by about 38% (Figure 1b, Table 1). Further increasing the duration of MAE to 10 min gradually decreased the extraction yield; the content of polyphenols extracted with 10-min MAE was similar to that with 1-min MAE. These findings showed that UAE and MAE had finished by 10 min and 5 min, respectively. The most efficient time of UAE resulted in 17% higher polyphenol yield than the most efficient time in MAE since the sample disruption by microwaves could have been less than that by ultrasonic treatment, as reported by SEM images of olive

pomace extracted with these methods [28]. In the present study, after 10 and 5 min of UAE and MAE, the TPC, TFC, and phenolic acid content were negatively correlated with the time of extraction ($r_s = (-0.31) - (-0.44)$; $p \leq 0.05$) (Figure S2). Although MAE and UAE partially break the polyphenol network into low molecular weight polyphenols, allowing intracellular bioactive compounds to be released in a short time, polyphenols can degrade if they are exposed to microwaves and ultrasound for excessive time periods. In the present study, the decrease in polyphenol yield with time period durations of UAE above 10 min and MAE above 5 min could be due to the production of free hydroxyl radicals, which allows highly active substances to break the chemical structure of polyphenols, especially when water content is high, and due to temperature increase [28]. Acoustic cavitation or high temperatures promote reactions of oxidation, polymerization, and decomposition, thus generating other products. Therefore, for example, a study by Qiao et al. [32] showed that four phenolic acids (protocatechuic acid, p-OH benzoic acid, vanillic acid, and p-coumaric acid) were stable, while three phenolic acids (caffeic acid, sinapic acid, and ferulic acid) were degraded by ultrasound. Regarding temperature, it should not have caused decomposition of polyphenols in the present study, since extractions were conducted at a temperature that did not exceed a safe value for thermolabile polyphenols [29].

3.1.2. Effect of Solid-to-Solvent Ratio on Polyphenol Recovery

The efficiency of recovering phenolic compounds from distillery stillage was about 17% higher (UAE) or about 14% higher (MAE) at a solid-to-acetone ratio of 1:15 than at 1:30 (*w*:*v*) (Figure 1a,b, Table 1). This can be explained by the fact that the strength of the solvent was so high that a smaller amount of solvent was sufficient for recovering phenolic compounds, while a larger amount diluted the sample. Additionally, it should be noted that, after establishing equilibrium in the phenolic compound recovery reaction, there is a steady state where an increase in the amount of the solvent does not affect recovery efficiency [33]. Therefore, in the present study, increasing the solvent volume did not improve the efficiency of phenolic compound extraction. A similar result during extraction of bioactive compounds from olive leaves was explained by differences in concentrations of these compounds between the interior and exterior of the olive leaf cell, which affected the mass transfer in extraction [34]. The fact that a lower acetone-to-solid ratio favored extraction yield is beneficial concerning both the extraction costs and the concentration and purification of extracts. However, to interpret the effect of solvent quantity on polyphenol recovery more comprehensively, the affinity of the solute for the solvent was determined, including behaviors attributed to hydrogen bonding and polar interactions (Section 3.1.3).

3.1.3. Effect of Solvent Concentration on Polyphenol Recovery

TPC and TFC decreased as acetone concentrations were changed in the following order: 60% > 80% > 40% > 100% (Figure 1a,b, Table 1). Using 60% acetone produced about 2-times higher polyphenol yield than using 100% acetone. The TPC, TFC, and phenolic acid content were strongly negatively correlated with the solvent concentration ($r_s = (-0.90) - (-0.97)$; $p \leq 0.05$) (Figure S2). Extraction procedures, including solvent characteristics, strongly affect the recovery of phenolic compounds, which dissolve better in polar organic solvents than in non-polar ones, due to the presence of hydroxyl groups in the polar solvents [28]. The addition of water to the solvent reduces the dielectric constant, increasing solvent diffusion and desorption of the solute from the sample. Therefore, in the present study, aqueous mixtures of acetone were more effective in recovering polyphenols than pure acetone. Similarly, 60% acetone proved the best solvent concentration for the recovery of phenolic compounds from brewers' spent grain (4.00 mg GAE/g DM) [35] and *Salsola tomentosa* (TPC of 20.37 mg GAE/g and TFC of 2.08 mg QUE/g) [36]. All these observations show that approaching the polarity of the solvent to the polarity of solutes, thus increasing the surface area of the solute–solvent contact, improves extraction yield [28]. This confirms the necessity of optimizing the solvent concentration for extracting particular compounds from plant materials.

To better explain the relationship between acetone concentration and polyphenol extraction yield, the Hansen Solubility Parameter (HSP) was employed to assess the solubility of phenolic compounds in acetone. The individual six phenolic acids that were detected in the extracts were selected for these calculations. The theory of the HSP is based on the cohesion energy between molecules, which is described in terms of dispersion forces (D), polarity (P), and the energy of intermolecular hydrogen bonds (H). Respective solubility parameters (δ_D, δ_P, and δ_H) were calculated based on molar volume (V_m) and group contributions to individual solubility parameters ($F_{D,i}$, $F_{P,i}$ and $F_{H,i}$), based on Hansen [37]. The affinity of phenolic acids for acetone and water was assessed based on the values of the total solubility parameters (δ_T) (Equations (3)–(6)) (Table 2).

$$\delta_D = \frac{\sum F_{D,i}}{V_m} \quad (3)$$

$$\delta_P = \frac{\sqrt{\sum F_{P,i}^2}}{V_m} \quad (4)$$

$$\delta_H = \frac{\sqrt{\sum F_{H,i}}}{V_m} \quad (5)$$

$$\delta_T^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \quad (6)$$

Table 2. Hansen solubility parameters of the solvents and the phenolic acids.

Compound	V_m (mL/mol)	δ_D	δ_P	δ_H	δ_T
water	18.06	15.5	16.0	42.3	47.8
acetone	74.08	15.5	10.4	7.0	19.9
ferulic acid	136.75	22.7	5.7	15.6	28.1
vanillic acid	119.25	22.6	6.5	16.6	28.8
p-coumaric acid	118.10	20.4	5.6	16.0	26.5
p-OH benzoic acid	94.60	22.9	7.0	17.8	29.8
syringic acid	151.28	21.2	5.8	15.4	26.8
sinapic acid	167.32	21.6	5.2	14.7	26.6

The affinity between the molecules is greater the smaller the difference between the δ_T values for each one [37]. The dispersion parameter (δ_D) was similar for all tested substances, meaning it did not bear a significant effect on the miscibility and solubility of these components. From Table 2 it can be concluded that the polarity (δ_P) and the hydrogen bond energy (δ_H) exhibited the largest effect on the affinity of the solutes for the solvents. The greatest differences were observed in the case of the energy of hydrogen bonds for water and phenolic acids, which suggests that, under normal conditions, water is the least compatible of the tested solvents for phenolic acid extraction. Therefore, in this study, mixtures of water and acetone at different concentrations were used as the solvent to determine the optimal solvent composition for the recovery of phenolic compounds. Therefore, it was necessary to determine the HSP values for each composition of the mixture.

In the case of multi-component solvents, the HSP of the solvent (δ_{mix}) is calculated from the volume fraction of the individual components in the solvent mixture (φ_i) and their contributions to the HSP of the mixture (δ_i). Finally, to determine the affinity of multi-component solvents (aqueous acetone solutions) for specific phenolic acids, the distance between the points representing the solute ($\delta_{D,A}$, $\delta_{P,A}$, $\delta_{H,A}$) and the solvent ($\delta_{D,B}$, $\delta_{P,B}$, $\delta_{H,B}$) was calculated, which represents the Hansen solubility parameter distance (R) (Equations (7) and (8)).

$$\delta_{mix} = \sum_i \varphi_i \delta_i \quad (7)$$

$$R = \sqrt{4(\delta_{D,A} - \delta_{D,B})^2 + (\delta_{P,A} - \delta_{P,B})^2 + (\delta_{H,A} - \delta_{H,B})^2} \quad (8)$$

Optimal selection of the components of the solvent mixture can improve the efficiency of phenolic acid recovery. Thus, the observed differences in the R parameter were dependent on the concentration of acetone in the solvent mixture (Figure 2).

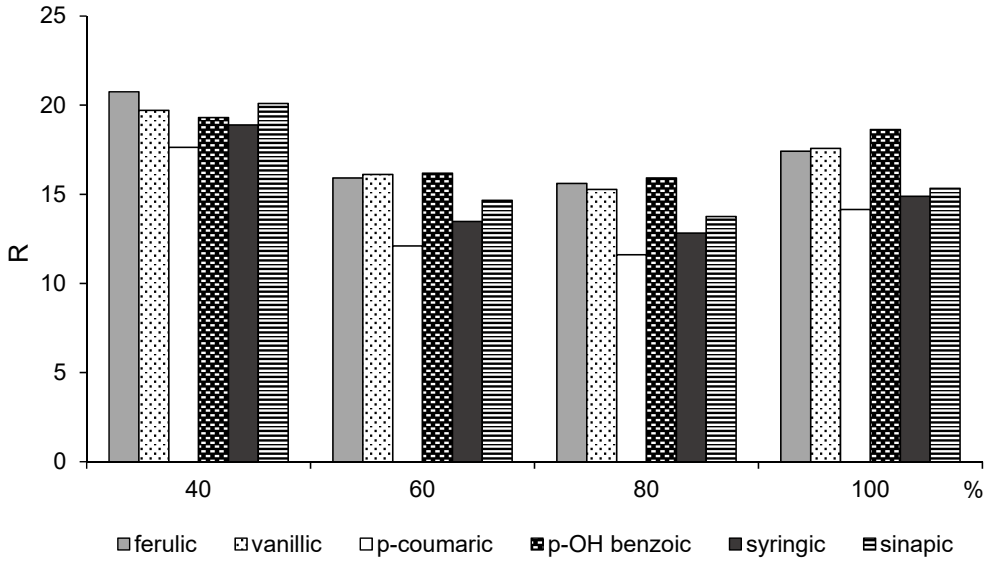


Figure 2. Hansen solubility parameter distances (R) between phenolic acids and 40%, 60%, 80%, and 100% acetone.

The R values for phenolic acids in aqueous acetone solutions revealed a non-monotonic trend with the minimum values of this parameter (12.11–16.18 and 11.61–15.92, respectively) at 60% and 80% (*v*:*v*) acetone (Figure 2). Low R values indicate high solubility as the interaction forces between the molecules of all components are similar. It was observed that acetone–water mixtures were the most effective solvents for extraction of the mixture of phenolic acids recovered from stillage as the phenolic acids that were extracted differ only slightly in their structure. This is because adding water to acetone increases the entropy of the mixture, and thus the structure of the solvent mixture becomes less ordered, which favors the interaction of phenolic acids with the solvent mixture [35]. Therefore, the HSP provides a simple method of predicting the solvent composition that will most efficiently recover bioactive compounds.

However, whereas the HSP analysis predicted that 60% and 80% aqueous solutions of acetone would recover phenolic acids with equal efficiency (Figure 2), the efficiency of TPC recovery was significantly higher with the 60% solution (Figure 1). This discrepancy resulted from the fact that the mixture of phenolic acids in the real sample created more complex natural products. The affinity of these compounds for solvents at different concentrations and the resulting selectivity of extraction are determined by various interactions between substrate components and the bioactive compounds, which is influenced by their chemical structure, overall composition, and the type of biomass from which the bioactive compounds are recovered [38].

3.1.4. pH and ST of the Extracts

The pH and ST of the extracts affect the stability and solubility of the bioactive compounds, which in turn affect the efficiency of extraction and the antioxidant activity of the extracts. Therefore, these two factors were monitored when optimizing the recovery of polyphenols. The pH of the UAE extracts ranged from 3.30 to 5.11, and that of the MAE extracts, from 3.36 to 4.98. Low pH significantly improves the recovery of phenolic compounds. Friedman and Jurgens [39] observed that in extracts with a pH of 1, the recovery of phenols from grape skins was three times higher than in those with a pH of 6 or 8. This is because phenolic compounds are more stable under acidic conditions than

under neutral conditions. In addition, under acidic conditions, phenolic compounds are released from the cell wall, resulting in high extraction efficiency [40].

In the present study, the ST of the extracts ranged from 22.63 to 29.90 mN/m (UAE) and from 22.57 to 29.68 mN/m (MAE). The ST values in the UAE and MAE extracts were lowest when using 60% acetone. This allowed easier penetration of acetone into the distillery stillage matrix, which increased the contact surface between the matrix and the solvent [34]. Therefore, the acidic pH and low ST of the extracts favored the transport of phenolic compounds into the solvent.

3.2. Phenolic Acids

3.2.1. Content of Phenolic Acids in the Extracts

All extracts displayed similar phenolic acid profiles, although the concentrations of free and bound phenolic acids varied. The average total content of phenolic acids (after hydrolysis) was 2.34 ± 0.23 μg/g DM. The concentrations of free phenolic acids ranged from 0.79 to 2.12 μg/g DM and from 0.59 to 1.78 μg/g DM with UAE, and from 0.72 to 1.89 μg/g DM and from 0.53 to 1.58 μg/g DM with MAE, at solid-to-solvent ratios of 1:15 and 1:30 (*w*:*v*), respectively. In the most efficient extraction variants of UAE and MAE, free phenolic acids accounted for 81–91% of the total phenolic acid content, respectively. The high level of free phenolic acids in the extracts resulted from the fact that distillery stillage is generated during the anaerobic processing of plant materials. In general, fermentation processes release bound compounds and increase their extractability from plant-origin materials [40].

In all extracts, the phenolic acid fraction consisted of six acids: p-OH benzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid (Figure 3). Vanillic, p-coumaric, and ferulic acids were detected with two characteristic peaks; the left peak indicated the unoxidized species (a) while the right peak indicated the more polar oxidized species (b). All three compounds contain a double bond in the carboxylic acid hydrocarbon chain, which is likely to be susceptible to oxidation [41]. Although the retention times of a particular phenolic acid differed slightly depending on whether it was in a UAE or an MAE extract (Figure 3), this did not prevent the identification of the acids. In addition, each peak was separated from the others, indicating that the extraction procedure was performed correctly and that one acid was not transformed into another one.

The recovery of free phenolic acids was highest when using 60% acetone during 10-min UAE at the solid-to-solvent ratio of 1:15 (*w*:*v*), with total values of 2.12 μg/g DM (Figure 4). In the case of MAE, the concentrations of phenolic acids were lower, amounting to 1.88 μg/g DM with 5-min extraction with 60% acetone at a solid-to-solvent ratio of 1:15 (*w*:*v*). The concentrations of individual free phenolic acids obtained in other variants of UAE and MAE are shown in Figure S1.

In the phenolic acid fraction of the extracts from distillery stillage, ferulic acid and p-coumaric acid predominated, with respective shares ranging from 40 to 46% and from 19 to 23%, respectively, depending on the extraction conditions (Figure 4). These acids were followed by vanillic and sinapic acids, with shares of 12–15% and 7–10%, respectively, and then by p-OH benzoic and syringic acids, which together accounted for about 4–6% of the total. Skrajda-Brdak et al. [42] discovered similar proportions of phenolic acids in wheat, rye, and oat grain. The composition and proportion between phenolic acids extracted with a particular solvent are related to the chemical structure of phenolic acids. Phenolic acids are classified as hydroxybenzoic acids or hydroxycinnamic acids [21]. Hydroxybenzoic acids include p-OH benzoic, vanillic, and syringic acids, while hydroxycinnamic acids include p-coumaric, ferulic, and sinapic acids; all these acids exist in the form of esters and glycosides. In the present study, the finding of predomination of ferulic and p-coumaric acids was attributed to the similarity in their chemical structures, namely the presence of a –CH=CH-COOH group. The C=C double bond participates in the stabilization of ferulic and p-coumaric acids, hence these acids were resistant to degradation [43]. The highest concentrations of ferulic and p-coumaric acids may be due to their location in the cell wall

in the cereals from which the alcohol was produced. These acids appear both in arabinose esterified bonds in hemicelluloses and etherified lignin bonds (while other phenolic acids are mostly esterified to lignin) [21]. This explains why ferulic and p-coumaric acids were released rather than degraded during alcoholic fermentation as might have been the case with other phenolic acids. Additionally, in other studies, it was noticed that vanillic acid may transform into ferulic acid due to physical and chemical interactions [20], which may result in a higher concentration of ferulic acid in extracts in the present study.

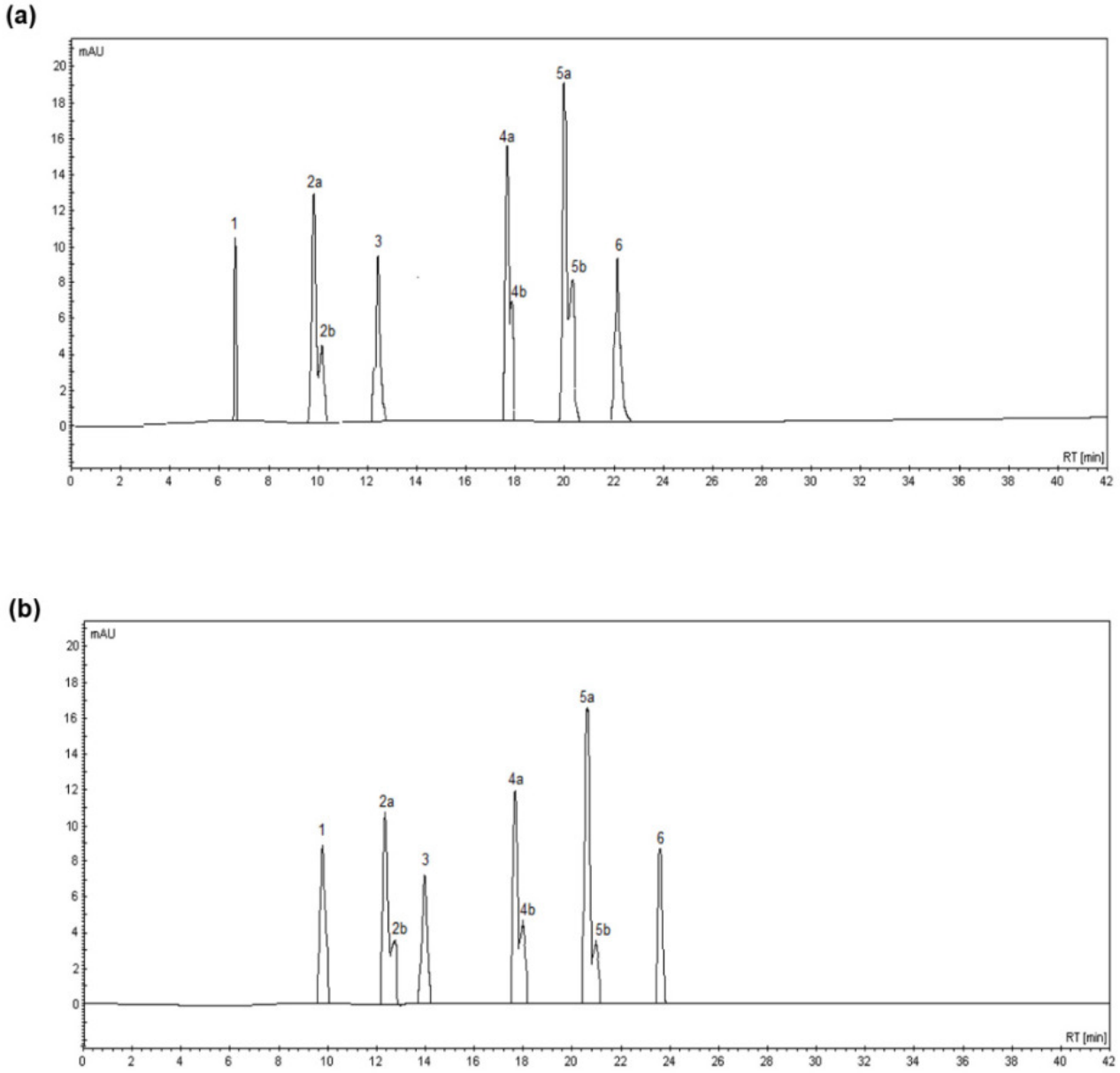


Figure 3. Chromatograms of phenolic acids in extracts from UAE (**a**) and MAE (**b**) with 60% acetone at a solid-to-solvent ratio of 1:15 (*w*:*v*). Peaks: (**1**) p-OH benzoic acid; (**2a**) vanillic acid; (**2b**) oxidized vanillic acid; (**3**) syringic acid; (**4a**) p-coumaric acid; (**4b**) oxidized p-coumaric acid; (**5a**) ferulic acid; (**5b**) oxidized ferulic acid; and (**6**) sinapic acid.

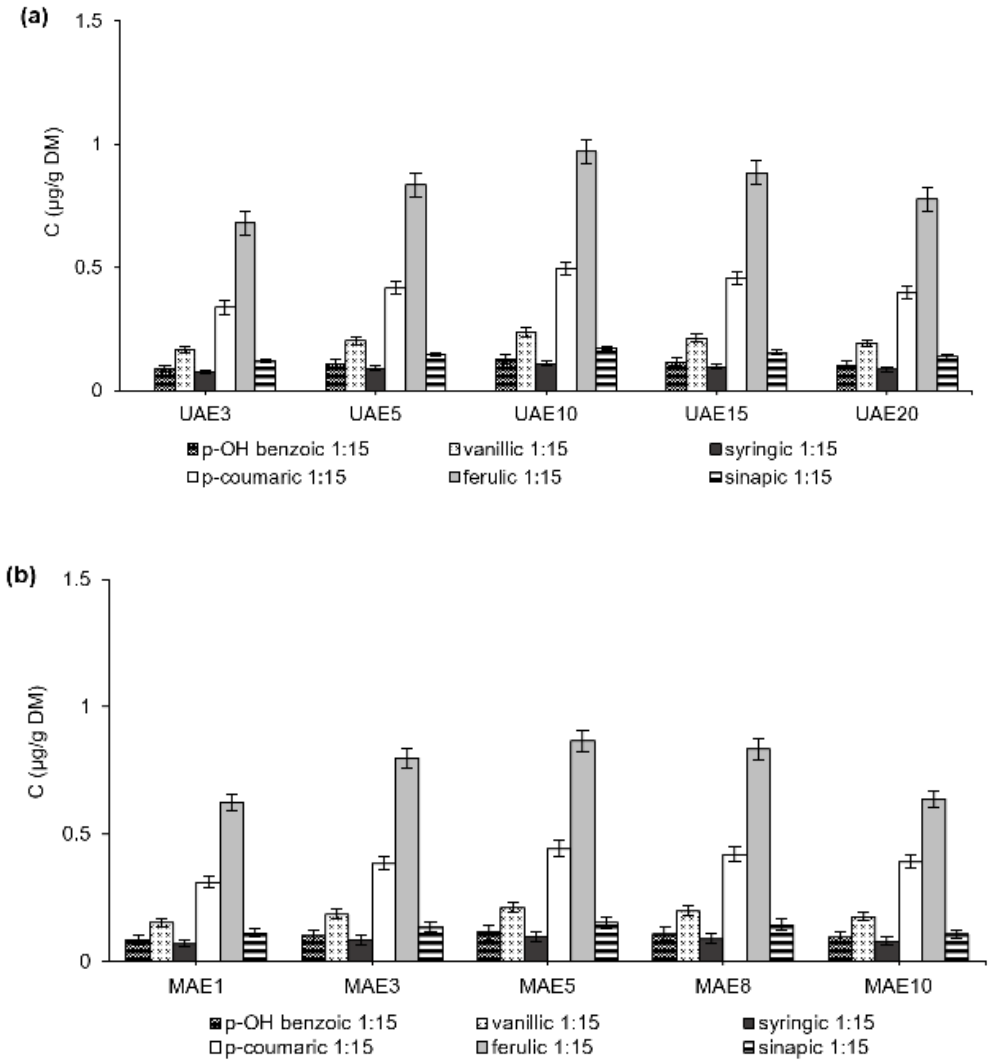


Figure 4. Concentrations of individual phenolic acids in extracts from UAE (**a**) and MAE (**b**) with 60% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time.

The phenolic acids that were extracted from the distillery stillage in the present study exhibit valuable properties. Ferulic acid protects against coronary artery disease, lowers serum and liver cholesterol levels, and increases sperm viability [44]. P-coumaric acid contains chemoprotective and antioxidant properties [45]. Vanillic acid is the major metabolic product of vanillin aldehyde, which exhibits antimicrobial, antimutagenic, and anti-carcinogenic effects [46]. Sinapic acid acts as a powerful oxidant scavenger, thus protecting cellular components [47]. P-OH benzoic acid has antimicrobial activity and is widely used as a preservative in food, cosmetic, and pharmaceutical products [32]. Syringic acid restores insulin sensitivity, increases glucose consumption, and is involved in carbohydrate metabolism [48].

3.2.2. Kinetics of UAE and MAE

The appropriate conditions for the rapid extraction of phenolic acids from distillery stillage were confirmed by the study of extraction kinetics. In Table 3, extraction rates are given, which were calculated for the ranges 0–10 min (UAE) and 0–5 min (MAE). For all experimental conditions, the determination coefficient R^2 was 0.99, indicating that the equations of the second-order kinetics adhered well to the subsequent experimental results. The content of total phenolic acids in the extracts increased with the maximal rates of

1.14 µg/(g DM·min) (UAE) and 3.93 µg/(g DM·min) (MAE) at a solid-to-solvent ratio of 1:15 (*w*:*v*) with 60% acetone. At each operating condition and for each phenolic acid, the extraction rate in MAE measured about 3 times higher than that of UAE. This may indicate that heat transfer was responsible to a higher extent for the rate of dissolution of solute than the cavitation effect. Moreover, ultrasound waves are considered to affect the extraction process but not diffusion through the solid particles [28]. However, when extracting bioactive compounds from olive pomace, the extraction rates were 1.1, 3.7, and 1.1 times higher in UAE than in MAE, for hydroxytyrosol, maslinic acid, and oleanolic acid, respectively [28]. These contradictory results suggest that different extraction methods should be optimized each time when extracting compounds from different types of waste material.

Table 3. Extraction rates for particular phenolic acids and the total phenolic acids. In the abbreviations used to refer to the series, the values after A show the solvent concentration; and finally, the solid-to-solvent ratio is given.

Type of Extraction	Extraction Rate (µg/(g DM·Min))						
	p-OH Benzoic	Vanillic	Syringic	*p*-Coumaric	Ferulic	Sinapic	Total Acids
UAE A40 1:15	0.047	0.085	0.038	0.178	0.348	0.062	0.758
UAE A60 1:15	0.070	0.130	0.058	0.249	0.539	0.095	1.142
UAE A80 1:15	0.054	0.098	0.045	0.206	0.403	0.071	0.878
UAE A100 1:15	0.034	0.062	0.028	0.131	0.255	0.045	0.557
UAE A40 1:30	0.038	0.069	0.032	0.145	0.284	0.051	0.620
UAE A60 1:30	0.065	0.103	0.055	0.191	0.458	0.089	0.958
UAE A80 1:30	0.049	0.079	0.040	0.166	0.306	0.064	0.703
UAE A100 1:30	0.026	0.049	0.023	0.089	0.188	0.036	0.412
MAE A40 1:15	0.151	0.272	0.125	0.570	1.118	0.199	2.435
MAE A60 1:15	0.250	0.453	0.206	0.850	1.846	0.328	3.929
MAE A80 1:15	0.180	0.330	0.150	0.687	1.342	0.238	3.417
MAE A100 1:15	0.099	0.180	0.081	0.375	0.732	0.129	1.594
MAE A40 1:30	0.116	0.211	0.095	0.442	0.860	0.153	1.875
MAE A60 1:30	0.306	0.400	0.214	0.715	1.728	0.339	3.647
MAE A80 1:30	0.179	0.294	0.152	0.619	1.138	0.238	2.618
MAE A100 1:30	0.079	0.154	0.071	0.275	0.581	0.111	1.272

3.3. Antioxidant Activity

To determine the usefulness of recovered phenolic acids, their antioxidant activity was determined. This activity depends on the type of substrate, the recovery procedures, and the cereal growing conditions since the active compounds are usually synthesized in response to stress, such as attack by microorganisms or strong UV radiation [46].

In the present study, the antioxidant activity of the extracts from the stillage was assessed employing ABTS, DPPH, and FRAP assays. Figure 5 displays the antioxidant activity of polyphenols extracted with UAE and MAE using 60% acetone. The antioxidant activity of extracts obtained with other variants of UAE and MAE is shown in Table S1.

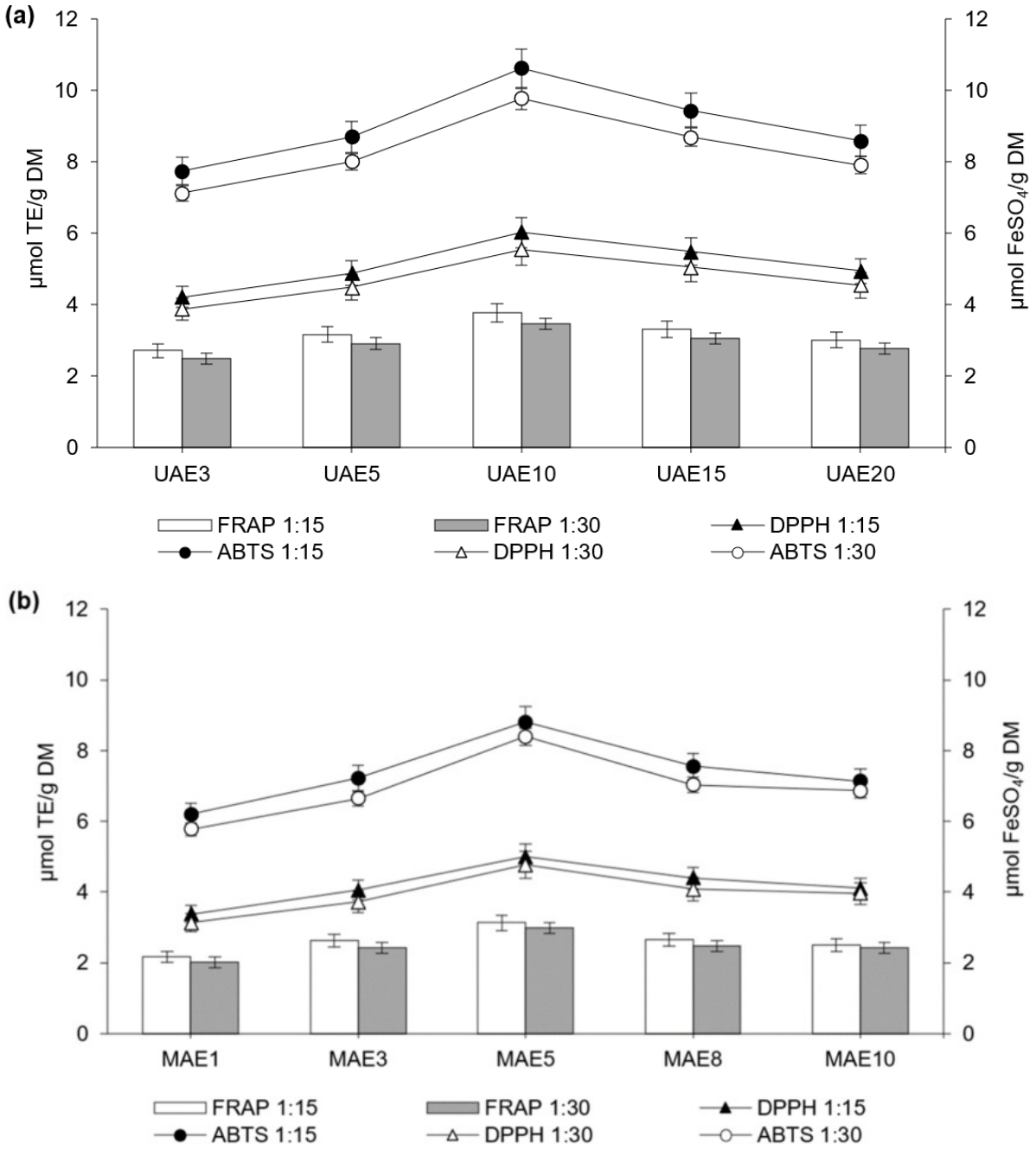


Figure 5. Antioxidant activity of the extracts from UAE (**a**) and MAE (**b**) with 60% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time.

The antioxidant activities of extracts obtained with 60% acetone were significantly higher than those of the extracts obtained with the other acetone concentrations. These activities correlated positively with the content of phenolic acids (Figure S2); therefore, it can be assumed that these compounds were responsible for the antioxidant properties of the extracts. Since the activity was strongly dependent on the content of phenolic acids, the activity of extracts obtained with 10-min UAE measured the highest, as indicated by ABTS (9.76–10.60 µmol TE/g DM), DPPH (5.54–6.03 µmol TE/g DM), and FRAP (3.47–3.77 µmol $FeSO_4$/g DM). Slightly lower values of antioxidant activity were obtained when MAE was used for 5 min, as indicated by ABTS (8.39–8.80 µmol TE/g DM), DPPH (4.77–5.00 µmol TE/g DM), and FRAP (2.98–3.13 µmol $FeSO_4$/g DM). Lengthening UAE beyond 10 min and MAE beyond 5 min decreased extract activity due to lower content

of phenolic acids in the extracts. This was not entirely consistent with other studies, in which only increases in extraction time above 45 min decreased the antioxidant activity of mung bean extract [29]. Kwaw et al. [30] observed that 15 min of UAE significantly increased (1.0–1.2 times) the phenolic content and antioxidant properties of mulberry juice. A similar trend was observed in the studies by Mehmood et al. [48], where it was noted that the antioxidant activity of compounds recovered from the butterfly pea flower was significantly higher (1.2–1.4 times) after UAE than after conventional recovery methods.

The results of this study revealed that ferulic and p-coumaric acids, as the acids comprising the highest content in 60% acetone extracts, are the components most responsible for the antioxidant activity of the distillery stillage extracts. Kikuzaki et al. [49] and Strazisar et al. [50] also reported strong antioxidant activity of these two acids. This property is attributed to their structural characteristics. The phenolic nucleus and unsaturated side chain in the structure of ferulic and p-coumaric acids can create stabilized phenoxy radicals, which account for their potent antioxidant activity [43]. The bioactive properties are affected by the size and polarity of the molecules, as well as the modification of the polarity of the substituents on the aromatic ring [51]. All acids with hydroxyl groups on the aromatic ring are highly active, therefore hydroxycinnamic acids are more active than hydroxybenzoic acids [52]. In the case of hydroxycinnamic acids, ferulic acid exhibits a higher antioxidant activity than p-coumaric acid. On the other hand, the presence of polar functional groups on an aliphatic side chain often reduces the antioxidant activity of aromatic compounds [51]. According to Wang and Wang [20], the presence of the carboxyl group reduces the activity to a greater extent than the presence of the hydroxyl group. For this reason, despite the similar structure, syringic acid is less active than ferulic acid, p-coumaric acid, and sinapic acid. The properties of sinapic acid derive from its carboxyl substituent and three other substituents. A similar effect can be observed when comparing the activity of vanillic and p-coumaric acids, which have similar molecular weights. However, p-coumaric acid is more biologically active since it has fewer substituents and a longer side chain than vanillic acid. However, despite knowledge regarding the activity of these individual compounds, real extracts should be investigated in terms of not only their phenolic acid composition but also in terms of their antioxidant activity. Natural extracts may be more beneficial than isolated compounds since the synergistic interactions of compounds can alter the bioactive properties of individual ingredients [50].

In the present study, in all extracts, the ABTS assay indicated significantly higher antioxidant activity than the DPPH assay, which was followed by the FRAP assay. However, values obtained in each of the assays exhibited a strong positive correlation with each other (Figure S2). Similarly, Yu et al. [11] found that the ABTS assay for wheat bread extract showed 10–20 times higher values than that indicated by the DPPH assay. Smuda et al. [8] discovered that the ABTS assay showed higher activity in wheat, rice, and maize by-products than the FRAP assay. One observation of this is that each assay bears a different mechanism, based on HAT or SET. The antioxidant activity of the phenolics is highly dependent on the molecule structure, namely the position and the number of hydroxyl groups. These two assays (ABTS and DPPH) are complementary and provide an abundance of information on the capability of phenolic acids to react with free radicals. The FRAP assay shows a different action since the reaction does not involve free radicals, but relies on the ability of antioxidants to reduce ferric iron (Fe^{3+}) to iron (Fe^{2+}). As an effect, employing all these assays provides a full picture of antioxidant properties of extracts that contain a mixture of bioactive compounds which can react with free radicals through various mechanisms.

3.4. Should UAE or MAE Be Chosen for Polyphenol Recovery?

Although the average content of polyphenols that were recovered from distillery stillage in the present study was lower than that from other substrates like wheat, rice, or maize wastes [44,45], the high availability of distillery stillage, which represents a waste

material, throughout the year renders the stillage a promising material for the recovery of polyphenols.

In the present study, UAE allowed for the 17% higher recovery yield of total polyphenols than MAE at the best-operating conditions in both methods. However, these results were obtained at 10-min UAE and 5-min MAE, which resulted from a 3 times higher extraction rate in MAE than in UAE. For these extraction times, UAE required 94 Wh of energy, while MAE required 33 Wh, which in the final economic assessment should bear a significant effect on the selection of the extraction method. Therefore, it can be concluded that although UAE yields higher content of polyphenols, MAE provides a more economical process owing to lower power input due to a shorter extraction time. However, to consider the implementation of these processes in a full industrial scale, other factors describing economic and environmental impact should be evaluated. Xie et al. [28], when comparing kinetic constants and thermodynamic parameters in UAE and MAE for extracting bioactive compounds from olive pomace, found that UAE was the greener and most effective technique of extraction. According to these authors, the E factor, which is defined as the mass ratio of waste to the desired product, was lower in UAE than in MAE by 6% and 13%, at 40 °C and 50 °C, respectively. In addition, energy consumption and corresponding carbon emissions were lower in UAE than in MAE by 14% and 11%, at 40 °C and 50 °C, respectively. When selecting the extraction method, the fact that UAE is employed at low temperatures should be also highlighted, which is particularly important in the extraction of polyphenols, which are temperature sensitive.

When evaluating the economic and environmental effects of extraction, the issue of extract purification should be considered. Effective methods of purifying natural bioactive compounds from plant extracts include solid-phase extractions via adsorption–desorption. This type of purification uses various solid phases such as silica gel, stationary phases for hydrophilic interaction liquid chromatography (HILIC) in reverse phase materials, and ion exchange/polymer resins [53]. The authors obtained the purity of 80–98% by weight after processing extracts of fruit and vegetables with macroporous resins. The isolation and purification processes of commercial phenolic compounds must be cost-effective, scalable, economically feasible, and capable of isolating valuable compounds from plant materials without significant degradation. For this purpose, multi-step processing has been proposed, for example, membrane separation combined with adsorption–desorption. It was observed that crude solvent extracts subjected to adsorption–desorption on macroporous resins maintained high antioxidant activity [53].

Apart from phenolic compounds, bioactive compounds in plant materials include other secondary metabolites such as antibiotics, mycotoxins, alkaloids, food colors, or plant growth factors. Solid–liquid extraction with the use of organic solvents and supported by ultrasound waves and microwaves may be used to recover these compounds [7]. However, bioactive compounds range from very polar to very non-polar. Therefore, when recovering bioactive compounds, the selection of the type and concentration of extraction solvent and extraction conditions plays an important role in the extraction efficiency.

4. Conclusions and Future Perspectives

This study showed that distillery stillage provides a valuable source of bioactive polyphenols that can be recovered via UAE and MAE with acetone. A short extraction time (10-min UAE or 5-min MAE) and a 60% acetone solution yielded the highest polyphenol content. Polyphenols were extracted more effectively with UAE, but the balance between the polyphenol yield and the energy consumption of the process proved better with MAE. The antioxidant activity of the extracts strongly correlated with both the total content of polyphenols and the content of individual phenolic acids, indicating that these substances contributed substantially to the bioactive properties of the extracts. A total of six different phenolic acids were recovered in mainly free forms, predominantly ferulic acid and p-coumaric acid. These findings indicate that the phenolic acids recovered from distillery stillage can potentially serve as valuable compounds, thus opening new possibilities

for profitable valorization of this waste and an adjustment of the work of distilleries to the assumptions of a circular economy. Future studies should develop the methods of purification and concentration of phenolic compounds to obtain products that will be applicable in the food, cosmetic, and pharmaceutical industries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph19052709/s1, Figure S1: Concentrations of individual phenolic acids in the extracts obtained with UAE (a–d) and MAE (e–h) with 40%, 60%, 80% and 100% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time; Figure S2: Correlations between TPC, TFC, phenolic acid content, antioxidant activities and extraction parameters with UAE (a,b) and MAE (c,d) at the solid-to-solvent ratio of 1:15 and 1:30 (*w*:*v*), respectively. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circles are proportional to the correlation coefficients. Values of Spearman's rank correlation coefficient above 0.6 are described as a strong correlation; t–extraction time, C–acetone concentration; Table S1: Antioxidant activities of the extracts obtained with UAE and MAE with 40%, 80% and 100% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time.

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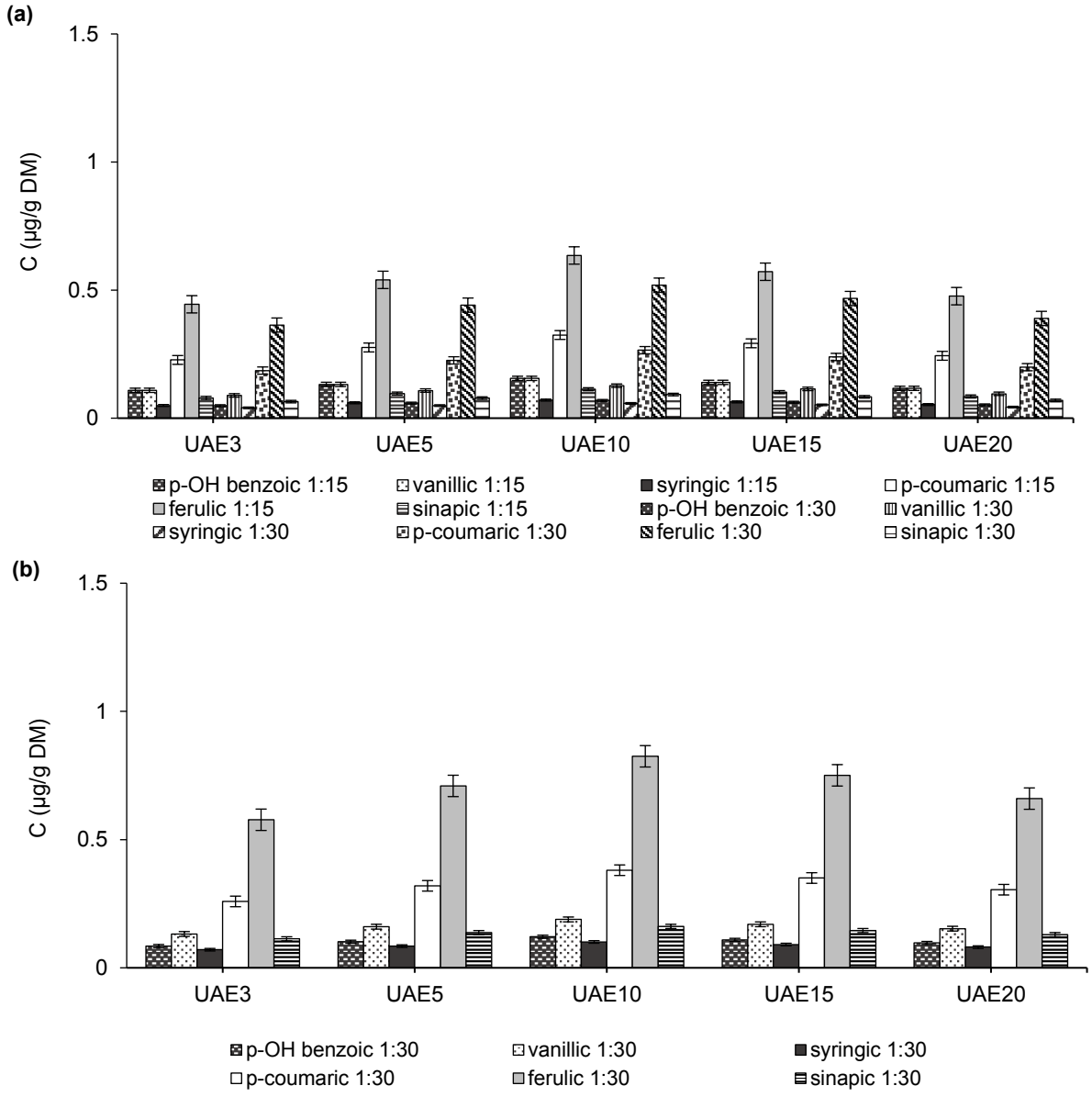
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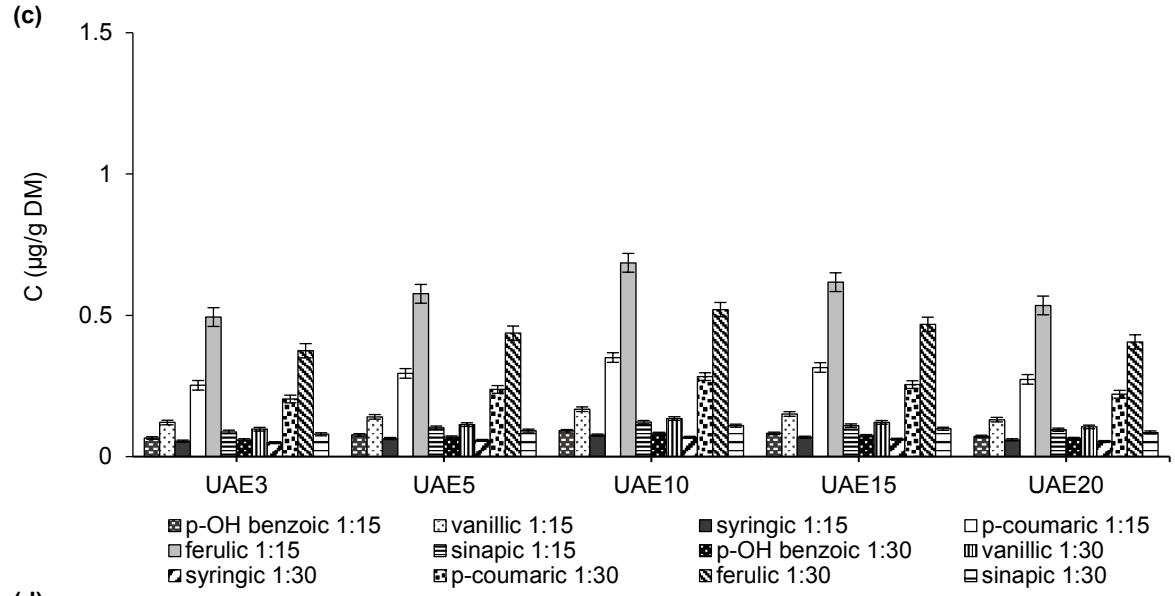
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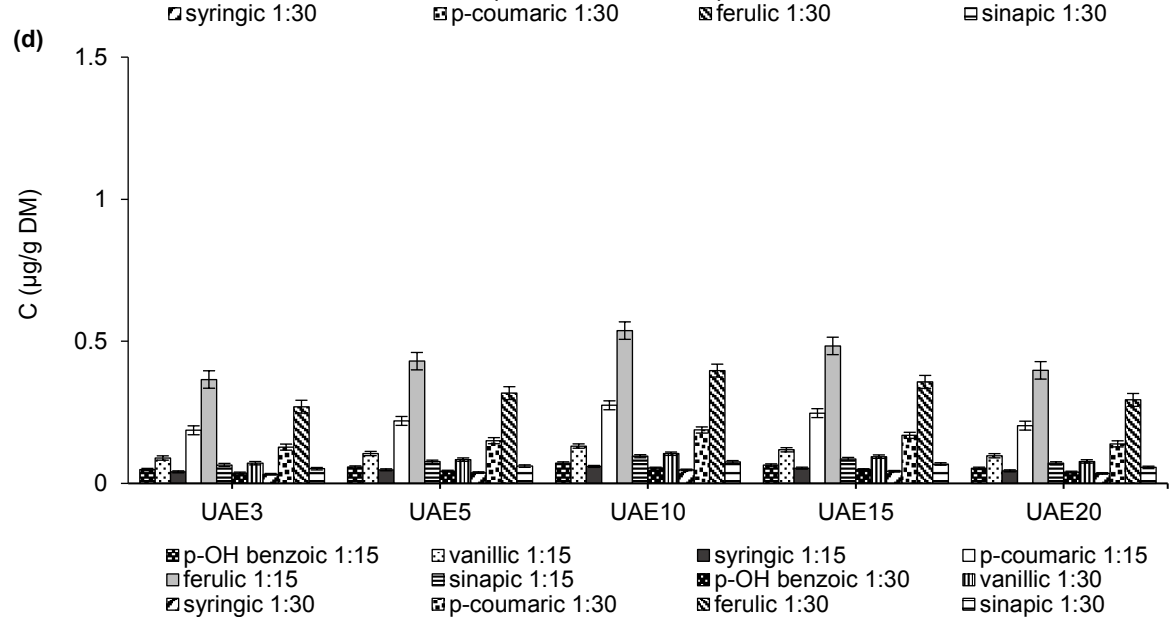
Processing of distillery stillage to recover phenolic compounds with ultrasound-assisted and microwave-assisted extractions

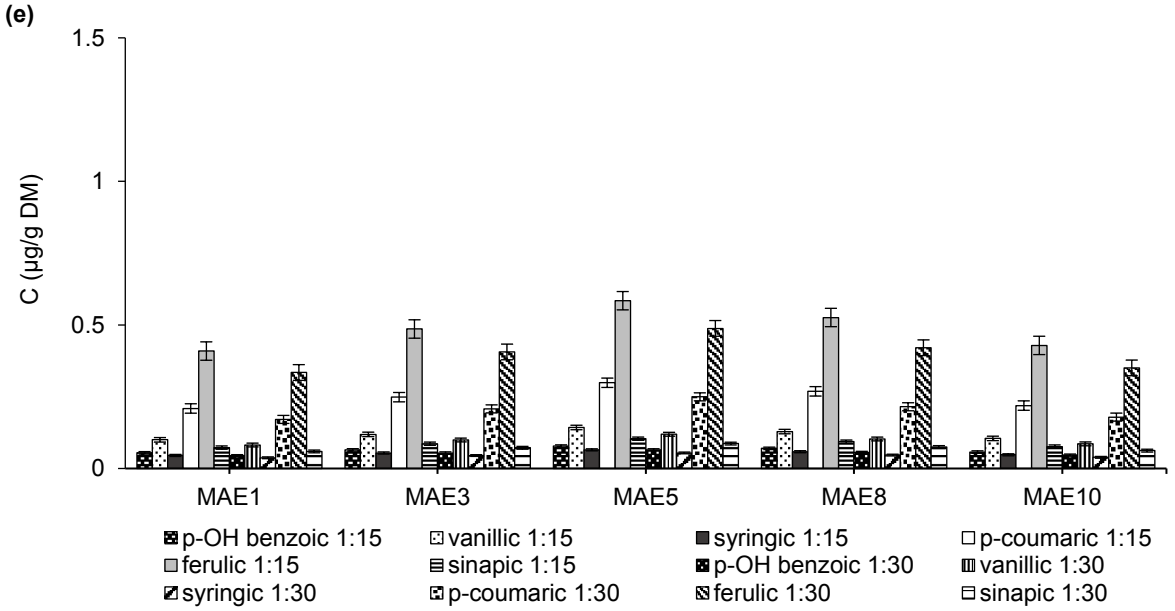
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Supplementary Material




(c)
C (µg/g DM)
1.5
1
0.5
0
UAE3
UAE5
UAE10
UAE15
UAE20
p-OH benzoic 1:15
vanillic 1:15
syringic 1:15
p-coumaric 1:15
ferulic 1:15
sinapic 1:15
p-OH benzoic 1:30
vanillic 1:30
syringic 1:30
p-coumaric 1:30
ferulic 1:30
sinapic 1:30


(d)
C (µg/g DM)
1.5
1
0.5
0
UAE3
UAE5
UAE10
UAE15
UAE20
p-OH benzoic 1:15
vanillic 1:15
syringic 1:15
p-coumaric 1:15
ferulic 1:15
sinapic 1:15
p-OH benzoic 1:30
vanillic 1:30
syringic 1:30
p-coumaric 1:30
ferulic 1:30
sinapic 1:30


(e)
C (µg/g DM)
1.5
1
0.5
0
MAE1
MAE3
MAE5
MAE8
MAE10
p-OH benzoic 1:15
vanillic 1:15
syringic 1:15
p-coumaric 1:15
ferulic 1:15
sinapic 1:15
p-OH benzoic 1:30
vanillic 1:30
syringic 1:30
p-coumaric 1:30
ferulic 1:30
sinapic 1:30

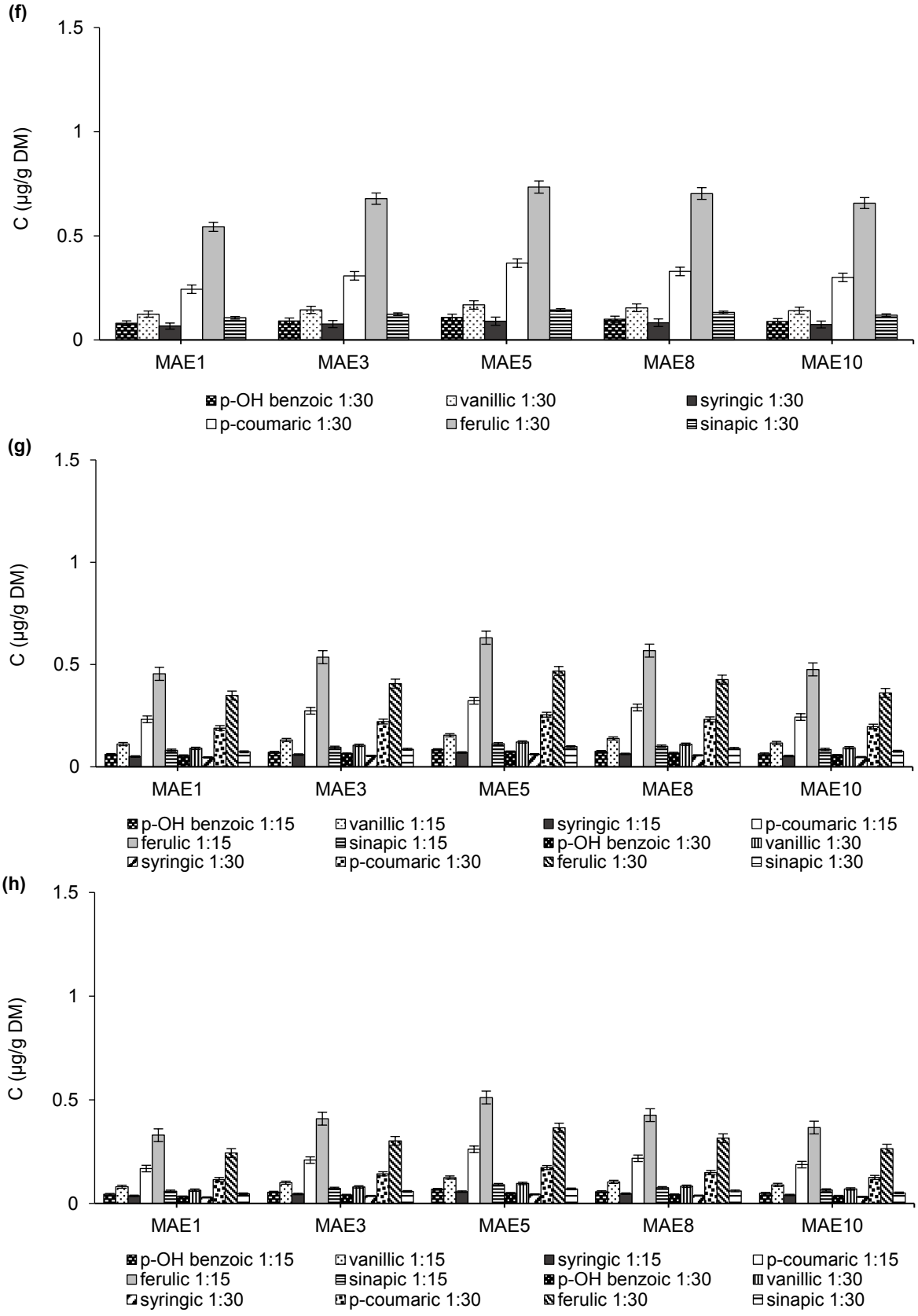
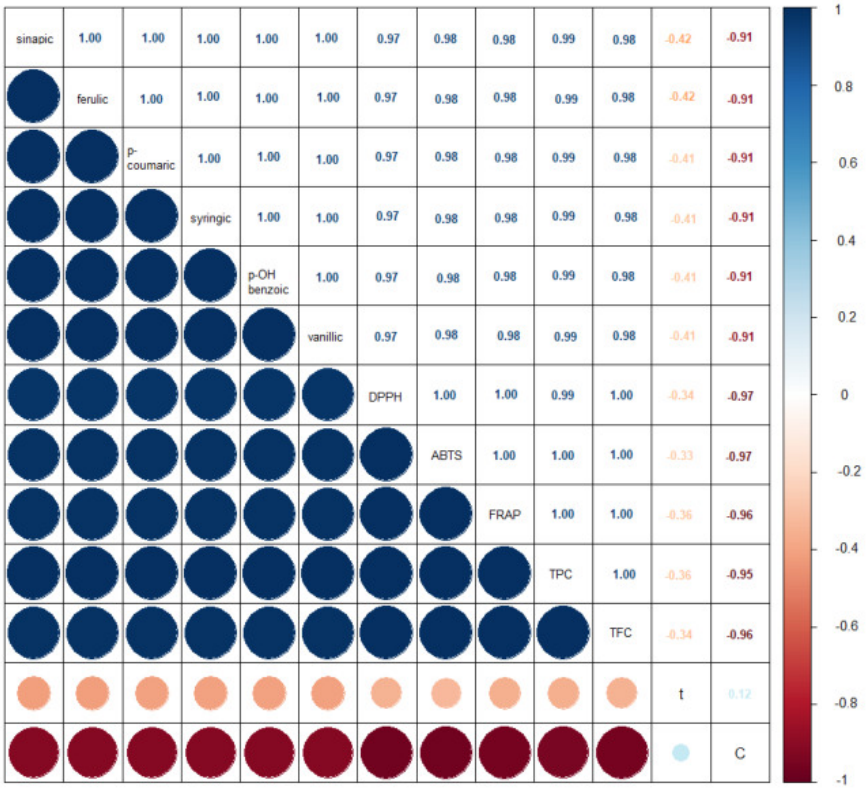
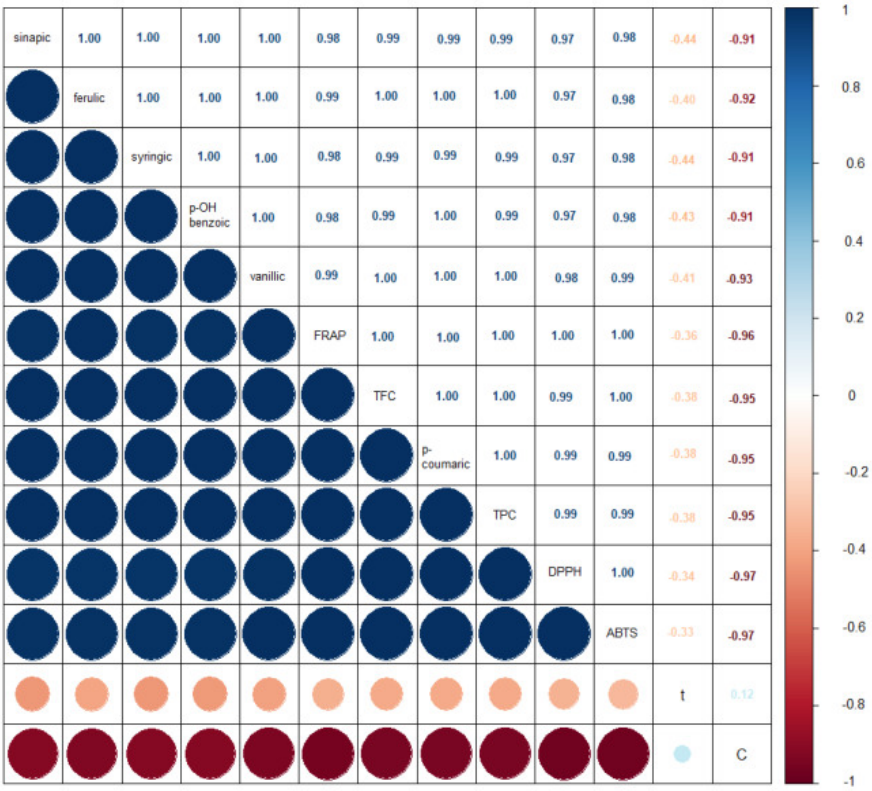


Figure S1. Concentrations of individual phenolic acids in the extracts obtained with UAE **(a, b, c, d**) and MAE **(e, f, g, h)** with 40%, 60%, 80% and 100% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time.

(a)


sinapic 1.00 1.00 1.00 1.00 1.00 0.97 0.98 0.98 0.99 0.98 -0.42 -0.91
ferulic 1.00 1.00 1.00 1.00 0.97 0.98 0.98 0.99 0.98 -0.42 -0.91
p-coumaric 1.00 1.00 1.00 0.97 0.98 0.98 0.99 0.98 -0.41 -0.91
syringic 1.00 1.00 0.97 0.98 0.98 0.99 0.98 -0.41 -0.91
p-OH benzoic 1.00 0.97 0.98 0.98 0.99 0.98 -0.41 -0.91
vanillic 0.97 0.98 0.98 0.99 0.98 -0.41 -0.91
DPPH 1.00 1.00 0.99 1.00 -0.34 -0.97
ABTS 1.00 1.00 1.00 -0.33 -0.97
FRAP 1.00 1.00 -0.36 -0.96
TPC 1.00 -0.36 -0.95
TFC -0.34 -0.96
t 0.12
C
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

(b)


sinapic 1.00 1.00 1.00 1.00 0.98 0.99 0.99 0.99 0.97 0.98 -0.44 -0.91
ferulic 1.00 1.00 1.00 0.99 1.00 1.00 1.00 0.97 0.98 -0.40 -0.92
syringic 1.00 1.00 0.98 0.99 0.99 0.99 0.97 0.98 -0.44 -0.91
p-OH benzoic 1.00 0.98 0.99 1.00 0.99 0.97 0.98 -0.43 -0.91
vanillic 0.99 1.00 1.00 1.00 0.98 0.99 -0.41 -0.93
FRAP 1.00 1.00 1.00 1.00 1.00 -0.36 -0.96
TFC 1.00 1.00 0.99 1.00 -0.38 -0.95
p-coumaric 1.00 0.99 0.99 -0.38 -0.95
TPC 0.99 0.99 -0.38 -0.95
DPPH 1.00 -0.34 -0.97
ABTS -0.33 -0.97
t 0.12
C
1
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0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

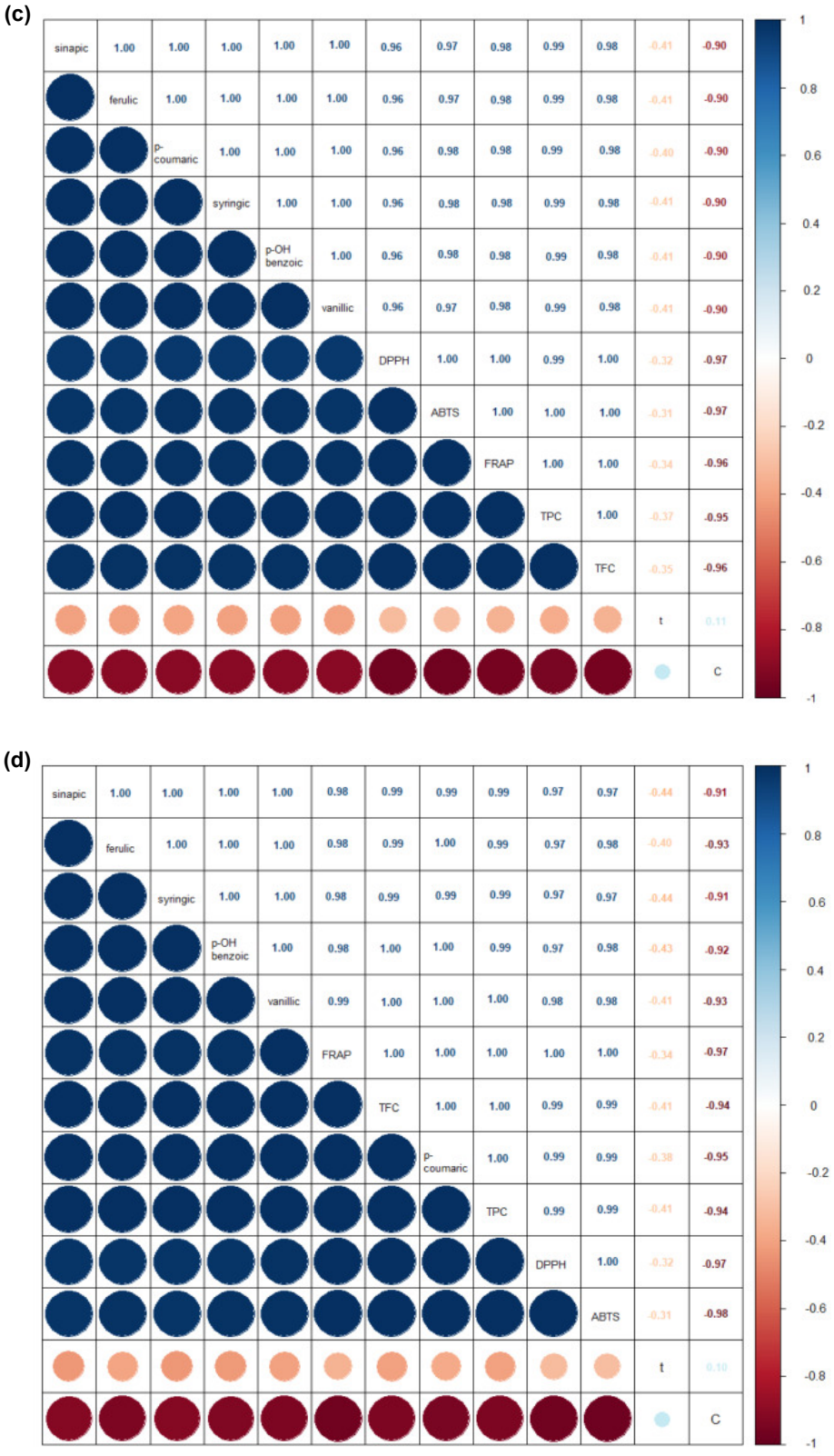


Figure S2. Correlations between TPC, TFC, phenolic acid content, antioxidant activities and extraction parameters with UAE **(a, b)** and MAE **(c, d)** at the solid-to-solvent ratio of 1:15 and 1:30 (w:v), respectively. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circles are proportional to the correlation coefficients. Values of Spearman's rank correlation coefficient above 0.6 are described as a strong correlation; t – extraction time, C – acetone concentration

Table S1. Antioxidant activities of the extracts obtained with UAE and MAE with 40%, 80% and 100% acetone. In the abbreviations used to refer to the series, the values after UAE and MAE indicate the extraction time.

Type of extraction	Antioxidant activity			Type of extraction	Antioxidant activity		
	DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)	ABTS (µmol TE/g DM)		DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)	ABTS (µmol TE/g DM)
UAE3 A40 1:15	3.61±0.57	2.11±0.23	6.44±0.68	MAE1 A40 1:15	3.00±0.62	1.75±0.27	5.35±0.87
UAE3 A80 1:15	4.09±0.48	2.30±0.28	6.42±0.66	MAE1 A80 1:15	3.39±0.55	1.91±0.23	5.33±0.86
UAE3 A100 1:15	2.48±0.37	1.37±0.15	4.55±0.58	MAE1 A100 1:15	2.06±0.42	1.14±0.19	3.78±0.76
UAE5 A40 1:15	4.39±0.41	2.56±0.27	7.82±0.78	MAE3 A40 1:15	3.64±0.48	2.12±0.24	6.49±0.55
UAE5 A80 1:15	4.77±0.44	2.69±0.26	7.49±0.83	MAE3 A80 1:15	3.96±0.51	2.23±0.22	6.22±0.49
UAE5 A100 1:15	2.92±0.29	1.62±0.34	5.36±0.73	MAE3 A100 1:15	2.48±0.39	1.37±0.11	4.55±0.63
UAE10 A40 1:15	5.16±0.62	3.01±0.42	9.20±0.94	MAE5 A40 1:15	4.39±0.35	2.56±0.26	7.82±0.72
UAE10 A80 1:15	5.68±0.66	3.20±0.40	8.92±0.91	MAE5 A80 1:15	4.71±0.43	2.66±0.31	7.40±0.77
UAE10 A100 1:15	3.65±0.34	2.02±0.29	6.70±0.61	MAE5 A100 1:15	3.03±0.31	1.68±0.29	5.56±0.63
UAE15 A40 1:15	4.64±0.43	2.71±0.25	8.28±0.76	MAE8 A40 1:15	3.72±0.28	2.17±0.24	6.62±0.45
UAE15 A80 1:15	5.11±0.61	2.88±0.23	8.03±0.83	MAE8 A80 1:15	4.09±0.32	2.30±0.11	6.42±0.59
UAE15 A100 1:15	3.28±0.35	1.82±0.16	6.03±0.56	MAE8 A100 1:15	2.63±0.46	1.45±0.14	4.82±0.29
UAE20 A40 1:15	3.87±0.38	2.26±0.19	6.90±0.71	MAE10 A40 1:15	3.21±0.25	1.87±0.26	5.73±0.47
UAE20 A80 1:15	4.43±0.42	2.50±0.21	6.96±0.72	MAE10 A80 1:15	3.72±0.32	2.10±0.24	5.84±0.44
UAE20 A100 1:15	2.70±0.19	1.49±0.13	4.96±0.55	MAE10 A100 1:15	2.27±0.29	1.26±0.22	4.16±0.36
UAE3 A40 1:30	3.32±0.27	1.94±0.18	5.93±0.59	MAE1 A40 1:30	2.76±0.33	1.61±0.08	4.92±0.64
UAE3 A80 1:30	3.76±0.35	2.12±0.16	5.91±0.61	MAE1 A80 1:30	3.05±0.28	1.72±0.14	4.78±0.59
UAE3 A100 1:30	2.28±0.29	1.26±0.09	4.19±0.44	MAE1 A100 1:30	1.85±0.19	1.02±0.09	3.39±0.38
UAE5 A40 1:30	4.04±0.43	2.35±0.22	7.19±0.38	MAE3 A40 1:30	3.35±0.23	1.95±0.13	5.97±0.42
UAE5 A80 1:30	4.39±0.46	2.47±0.13	6.89±0.63	MAE3 A80 1:30	3.77±0.26	2.13±0.18	5.93±0.78
UAE5 A100 1:30	2.68±0.24	1.49±0.11	4.93±0.52	MAE3 A100 1:30	2.31±0.18	1.28±0.24	4.24±0.55
UAE10 A40 1:30	4.75±0.60	2.77±0.16	8.46±0.68	MAE5 A40 1:30	4.08±0.44	2.38±0.28	7.28±0.49
UAE10 A80 1:30	5.22±0.43	2.94±0.23	8.20±0.73	MAE5 A80 1:30	4.33±0.46	2.44±0.25	6.81±0.38

UAE10 A100 1:30	3.36±0.34	1.86±0.17	6.16±0.69	MAE5 A100 1:30	2.78±0.55	1.54±0.15	5.11±0.69
UAE15 A40 1:30	4.27±0.32	2.49±0.28	7.62±0.75	MAE8 A40 1:30	3.46±0.37	2.02±0.17	6.17±0.54
UAE15 A80 1:30	4.70±0.46	2.65±0.34	7.38±0.78	MAE8 A80 1:30	3.81±0.31	2.15±0.09	5.98±0.23
UAE15 A100 1:30	3.02±0.26	1.67±0.21	5.54±0.52	MAE8 A100 1:30	2.51±0.25	1.39±0.16	4.60±0.39
UAE20 A40 1:30	3.56±0.22	2.08±0.29	6.35±0.33	MAE10 A40 1:30	2.92±0.21	1.70±0.18	5.21±0.44
UAE20 A80 1:30	4.07±0.38	2.30±0.31	6.40±0.29	MAE10 A80 1:30	3.42±0.16	1.93±0.21	5.38±0.62
UAE20 A100 1:30	2.48±0.36	1.38±0.05	4.56±0.34	MAE10 A100 1:30	2.06±0.13	1.17±0.08	3.87±0.41

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Attachment 4.

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Do	Wioleta Mikucka
Odpowiedź do	Apostolos P Kyriazopoulos
Data	Pn 14:46

CC: apkyriaz@fmenr.duth.gr, jmainali@stetson.edu

Ms. Ref. No.: JEMA-D-22-05107R1
Title: Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions
Journal of Environmental Management

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Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions

--Manuscript Draft--

Manuscript Number:	JEMA-D-22-05107R1
Article Type:	Research Article
Keywords:	distillery by-products; Hansen Solubility Parameters; phenolic antioxidants; Principal Component Analysis
Corresponding Author:	Wioleta Mikucka POLAND
First Author:	Wioleta Mikucka
Order of Authors:	Wioleta Mikucka
	Magdalena Zielinska
	Katarzyna Bulkowska
	Izabela Witonska
Abstract:	An increasing understanding of the negative environmental effects of waste discharges has made valorization of distillery by-products to recover added-value compounds a sound option for distillery stillage management. In this study, the recovery of bioactive compounds, i.e. polyphenols, from distillery stillage was performed by microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and conventional solid-liquid extraction (CSLE) to investigate the effects of extraction time, the concentration of ethyl acetate (EA) in a solvent mixture with ethanol and water, and solid-to-solvent ratio on the recovery yield and antioxidant activity of the extracts. The highest yields of total polyphenol content (TPC) (3.73 mg gallic acid equivalent/g) and phenolic acid content (2.51 μg/g) were obtained with 8-min MAE with 70% EA . MAE provided 1.2- and 1.4-times higher yield of phenolic acids and 1.2- and 1.6-times higher antioxidant activity than UAE and CSLE, respectively . Due to the approximately 3-times higher rate of extraction, the ratio between energy consumption and extraction yield was better in MAE than in UAE. Principal component analysis (PCA) showed that the antioxidant activity of the extracts was positively correlated with TPC and phenolic acid content. Six phenolic acids that were identified were present mainly in their free forms (up to 95% of the total), with a predominance of ferulic (up to 0.80 μg/g) and p -coumaric (up to 0.72 μg/g) acids.
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	Andrei Stanishevsky astan@uab.edu

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Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions

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*Corresponding author: wioleta.mikucka@uwm.edu.pl

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DISTILLERY STILLAGE

ETHYL ACETATE

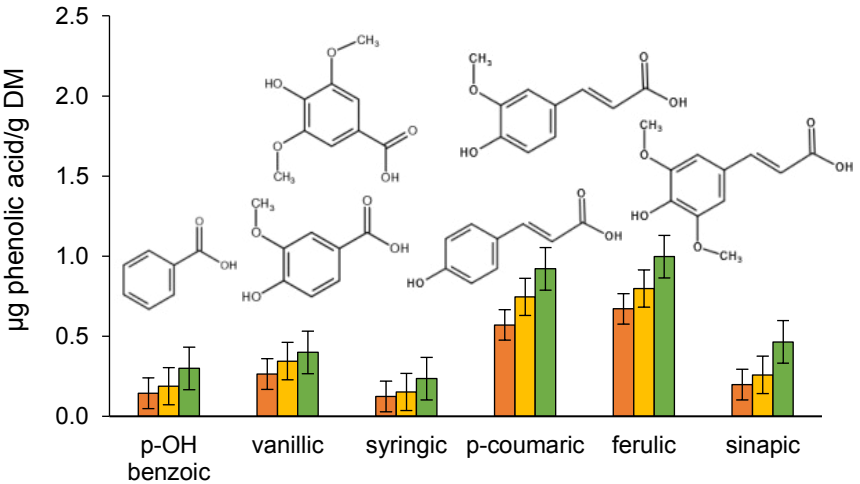
POLYPHENOL EXTRACTION

CONVENTIONAL

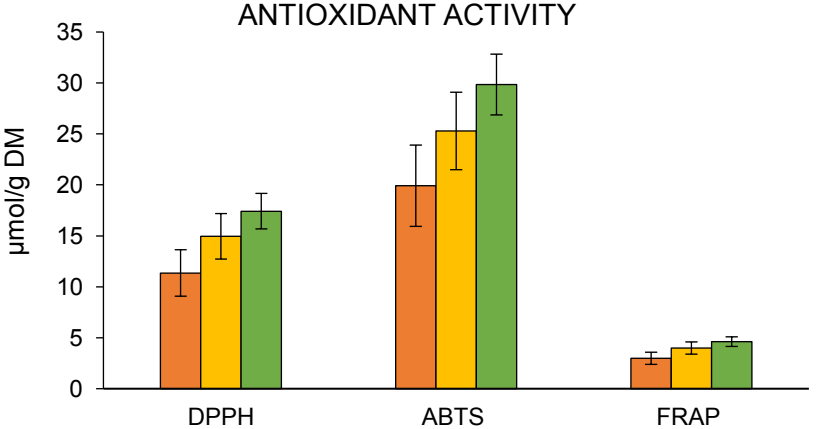
ULTRASOUND

MICROWAVE

EXTRACTED PHENOLIC ACIDS



ANTIOXIDANT ACTIVITY



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Highlights

- Distillery stillage is a source of high-value bioactive compounds.
- Six phenolic acids were recovered; ferulic acid and *p*-coumaric acid predominated.
- Recovered phenolic acids were present mainly in the free form.
- Phenolic acid recovery rate 3-times higher with microwaves than with ultrasounds.
- Antioxidant activity of the extracts correlated with the phenolic acid content.

Revised manuscript (Clean version) Click here to view linked References

Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions

Abstract

An increasing understanding of the negative environmental effects of waste discharges has made valorization of distillery by-products to recover added-value compounds a sound option for distillery stillage management. In this study, the recovery of bioactive compounds, i.e. polyphenols, from distillery stillage was performed by microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and conventional solid-liquid extraction (CSLE) to investigate the effects of extraction time, the concentration of ethyl acetate (EA) in a solvent mixture with ethanol and water, and solid-to-solvent ratio on the recovery yield and antioxidant activity of the extracts. The highest yields of total polyphenol content (TPC) (3.73 mg gallic acid equivalent/g) and phenolic acid content (2.51 µg/g) were obtained with 8-min MAE with 70% EA. MAE provided 1.2- and 1.4-times higher yield of phenolic acids and 1.2- and 1.6-times higher antioxidant activity than UAE and CSLE, respectively. Due to the approximately 3-times higher rate of extraction, the ratio between energy consumption and extraction yield was better in MAE than in UAE. Principal component analysis (PCA) showed that the antioxidant activity of the extracts was positively correlated with TPC and phenolic acid content. Six phenolic acids that were identified were present mainly in their free forms (up to 95% of the total), with a predominance of ferulic (up to 0.80 µg/g) and *p*-coumaric (up to 0.72 µg/g) acids.

Keywords: distillery by-products; Hansen Solubility Parameters; phenolic antioxidants; Principal Component Analysis

1. Introduction

Along with the development of the agri-food industry, solutions to manage a large amount of wastes produced by this sector should be also developed. Distilleries are an example of an industry generating huge amounts of by-products. In 2020, around 117 billion liters of alcohol were produced worldwide (Sonnichsen, 2020). About 88% of the raw plant materials used to produce alcohol are processed into waste which is termed distillery stillage and is characterized by a high content of difficult-to-degrade organic substances, nitrogen, and melanoidins that contain dark brown pigments (Kharayat, 2012).

In the concept of sustainable management, the search for various compounds of commercial importance that can be recovered from waste has started to be a key concern in not only reducing the pollution generated by the agri-food industry but also contributing to its economic competitiveness (Tapia-Quirós et al., 2022a). Raw plant materials used in the production of alcohol contain bioactive compounds such as polyphenols that include flavonoids and non-flavonoids (e.g. phenolic acids) (Shahidi and Ambigaipalan, 2015). Phenolic acids are hydroxy derivatives of benzoic or cinnamic acids and are present in plant materials in free or bound forms, or as free esters. Polyphenols exhibit antioxidant, antibacterial, antiviral, anticancer, and anti-inflammatory properties (Shahidi and Ambigaipalan, 2015). Because of the wide use of antioxidants in various industries (Canadas et al., 2020), the growing concern about the use of synthetic antioxidants and increasing consumer awareness of the health benefits of consuming natural antioxidants, research on phenolic acids has received special attention recently.

Apart from generating secondary streams of added-value compounds, the recovery of polyphenols positively affects subsequent anaerobic biological processes of waste utilization (Mikucka and Zielinska, 2022). For the recovery, the use of ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), mechanical stirring extraction or subcritical

water extraction were used (Tapia-Quirós et al., 2020; Montenegro-Landívar et al., 2021a; Mikucka et al. 2022b). Because polyphenols differ in their chemical structure and interactions with other components, selecting the optimal extraction process is a very important aspect of recovery. In addition, hydrolysis may affect the concentration and profile of recovered phenolic acids. Alkaline hydrolysis resulted in higher contents of *p*-coumaric, vanillic, and sinapic acids as compared with acidic hydrolysis when an inverse dependence was observed for syringic acid (Verma et al., 2009). The amount of gallic acid obtained after acid hydrolysis of grape skins without ultrasounds was 4.5-times higher than after extraction without hydrolysis but assisted by ultrasounds (Mazza et al., 2020). In addition to keeping the recovery yield at a high level, maintaining the functionality and quality of extracted compounds is important. For example, although the polyphenol yield from citrus peels using MAE was higher by about 16% than that when using UAE, the antioxidant activity was about 28% lower (Nayak et al., 2015). Because the antioxidant activity is affected by the composition (type of phenolic acids) and solubility of the antioxidants, the partition coefficient and solvent polarity, and the material from which phenolic compounds were recovered, its measurement should follow each extraction.

Although ethyl acetate (EA) is very selective for extracting low– and medium–molecular-weight phenolic molecules such as phenolic acids (Lesage-Meessen et al., 2001) and the antioxidant activity of EA extract was comparable to that of the synthetic ascorbic acid (Jayasiri et al., 2021), the number of studies on the use of non-toxic EA is limited in comparison to conventional solvents. Because of a lower boiling point of EA compared to ethanol (EtOH) or water, the content of phenolic compounds recovered from herbs decreased in the following order depending on the solvent used: EA > EtOH > hexane > water (Jayasiri et al., 2021). EA extracts had higher antioxidant activity than EtOH, acetone, and methanol extracts (Nayak et al., 2015). EA extracts of the plant had significantly higher polyphenol

content than EtOH, methanol, water and acetone extracts; however, the antimicrobial activities of EA, EtOH and methanol extracts were similar (Prabakaran et al., 2018). On the other hand, when extracting polyphenols from cornsilk powder, the polyphenol content in EA extracts was above 13 times lower and the antioxidant activity was 2 times lower than those in EtOH extracts (Nurhanan and Wan Rosli, 2012). The recovery of compounds depends above all on the solvent's polarity. Antioxidant compounds such as phenolic acids are preferably dissolved in solvents of a similar polarity. Polar solvents are usually considered to be better than non-polar solvents; therefore, the addition of water or another polar solvent (e.g. alcohol) improves the polarity of the solvent and hence the extraction efficiency (Xie et al., 2019). Therefore, to increase the efficiency of polyphenol recovery, various combinations of solvent mixtures should be investigated to improve the solvation power.

So far, polyphenols have been recovered from such raw materials as fruits, vegetables, and cereals (Garcia-Salas et al., 2010; Skrajda-Brdak et al. 2019; Montenegro-Landívar et al., 2021b), and from such wastes as olive pomace, winery residues, cereal by-products, pomegranate peel, citrus waste, other than distillery stillage (Tapia-Quirós et al., 2022b; Yu et al., 2013; Selvakumar et al., 2021; More and Arya, 2019; Yadav et al., 2022). Based on the studies cited above, it can be concluded that the recovery of phenolic compounds should be optimized for each type of waste material in terms of the extraction conditions and the solvent types since these compounds are usually combined with sugars, fatty acids, or proteins (Kumar and Goel, 2019). Because the reports on polyphenol recovery from distillery stillage are scarce, the key novelty of this study is to determine the potential of distillery stillage as a source of natural antioxidants that may be extracted with non-toxic solvents (EA in mixtures with EtOH and water) by CSLE, UAE, and MAE. The objective of this study was to determine the effects of the extraction technique, EA concentration, solid-to-solvent ratio, and

total extraction time on the TPC and TFC in the extracts, the types and content of free and bound phenolic acids, the extraction kinetics, and the antioxidant activity of the extracts.

2. Materials and Methods

2.1. Characteristics and preparation of distillery stillage

The distillery stillage used in the study was a by-product of the full-scale production of concentrated crude ethyl alcohol from cereals (including wheat and rye) in a distillery located in Poland ("53°30′00″ N, 20°10′19″ E"). Distillery stillage was characterized by: 47.0±5.3 g COD/L (Chemical Oxygen Demand), 4.345±0.005 g N_{total}/L, 0.280±0.002 g P_{total}/L, 0.789±0.003 g VFAs/L (Volatile Fatty Acids), and the moisture of 93.6±2.8%. After transport to the lab, the distillery stillage was frozen, freeze-dried (STERIS Lyovac GT2 freeze dryer with a Leybold Trivac vacuum pump, Germany) for 12 h, at -6°C and 8 Pa, and then stored. After drying, the moisture of the stillage was 9.8±0.9%.

2.2. Procedures of extraction

CSLE, UAE and MAE used pure EA (EA100), EA:water (90:10, v:v) (EA90), EA:EtOH:water (80:10:10, v:v:v) (EA80), EA:EtOH:water (70:20:10, v:v:v) (EA70), and EA:EtOH:water (60:30:10, v:v:v) (EA60). The extractions were performed with solid-to-solvent ratios of 1:15 (w:v) and 1:30 (w:v). CSLE was performed under shaking conditions (Orbital Shaker, R05 GERHARDT, Poland) at 150 rpm for 15, 30, 45, 60, and 90 min for three extraction cycles at room temperature. For UAE, an ultrasonic bath (InterSonic IS-5.5, Poland) with a 25 kHz frequency and 100% amplitude was used at room temperature in a continuous mode for 3, 5, 10, 15, and 20 min. MAE was performed in a microwave oven (MARSXpress 240/50 CEM, USA) at 400 W and 50°C for 1, 3, 5, 8, and 10 min. After CSLE, UAE, and MAE, the samples were centrifuged (Centrifuge MPW-380, Poland) for 10

min at 10,000 rpm to obtain the final extracts. The extraction conditions were based on the studies by Mikucka et al. (2022a).

2.3. Analytical methods

In distillery stillage samples, the concentrations of COD (dichromatic method) and VFA (distillation method; concentration expressed as acetic acid) (APHA, 1992), and P_{total} and N_{total} (LCK350 and LCK338 cuvette tests, respectively, Hach Lange GMBH, Germany) were measured. In the final extracts, the pH was measured with a HI 221 pH meter (HANNA Instruments, Romania). The surface tension of the solvents was measured on a plate with a K100C tensiometer (Germany) at room temperature, using KRUSS Laboratory Desktop. Two measurements per sample have been conducted.

2.3.1. TPC and TFC

In the extracts, TPC (Singletion et al., 1999) and TFC (Quettier-Deleu et al., 2000) were measured. A UV-Vis spectrophotometer (Varian Cary 50 Scan UV-Visible, Australia) was used. TPC was expressed as mg gallic acid equivalent (GAE) per g dry mass (DM). TFC was expressed as mg of Quercetin Equivalents (QUE) per g DM.

2.3.2. Content of phenolic acids

The contents of free phenolic acids in the extracts and total phenolic acids in distillery stillage (released by acidic hydrolysis) were measured with HPLC, according to Mikucka et al. (2022a). For hydrolysis, 1 g of the freeze-dried sample was mixed with 20 mL of 2M NaOH for 4 hours. The samples were acidified to pH 2 with 6M HCl and centrifuged for 10 min at 10,000 rpm. The supernatants were extracted four times using 20 mL of diethyl ether. The diethyl ether extracts were dried by a rotary vacuum evaporator and re-dissolved in 1 mL

of methanol. Chromatographic separation was performed by an HPLC (Varian, Australia) fitted with a UV-Vis detector equipped with a Supelcosil C18 column (150 mm × 4.6 mm, 5 μm) with the column flow rate set to 1 mL/min, and the temperature set to 35°C. The mobile elution phase was acetonitrile/formic acid (99.85:0.15, v:v) (eluent A) and water/formic acid (99.85:0.15, v:v) (eluent B). Phenolic acids were separated for 42 min at a gradient elution flow rate of 1 mL/min with the following multistep gradient: 0–18 min, 1–96% B; 18–35 min, 96–82% B; 35–40 min, 82–75% B. The analysis used wavelengths of 260 nm (p-OH-benzoic, vanillic, syringic acids) at 0–16.50 min and 320 nm (*p*-coumaric, ferulic, sinapic acids) till the end. All phenolic acid standards (95–99% purity, Sigma-Aldrich, USA) were prepared at a concentration of 0.1 mg/mL.

2.3.3. Antioxidant activity of the extracts

ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2, 2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidant power) assays were used. The ABTS, DPPH and FRAP were measured by Re et al. (1999), Moure et al. (2001) and Benzie and Strain (1996), respectively. ABTS and DPPH were expressed as μmol Trolox equivalent (TE)/g DM. FRAP was expressed as μmol $FeSO_4$/g DM. UV-Vis spectrophotometer (Varian Cary 50 Scan, Australia) was used.

2.4. List of reagents

In the experiments, the following reagents were used: ethyl acetate (99.8% purity, Sigma-Aldrich, USA), ethanol (99.8%, Chempur, Poland), gallic acid (≥98%, Sigma-Aldrich, USA), quercetin (≥95%, Sigma-Aldrich, USA), Folin-Ciocalteu reagent (Sigma-Aldrich, USA), methanol (99.9%, Merck, Germany), diethyl ether (99.5%, Chempur, Poland), acetonitrile (99.9%, Sigma-Aldrich, USA), formic acid (98–100%, Merck, Germany), water

for HPLC (Sigma-Aldrich, USA), ABTS (≥98%, Sigma-Aldrich, USA), DPPH (≥97%, Sigma-Aldrich, USA), FRAP (≥99%, Sigma-Aldrich, USA), Trolox (97%, Sigma-Aldrich, USA), $FeSO_4$ (≥99%, Merck, Germany).

2.5. Calculation and statistical methods

Hansen Solubility Parameter (HSPs) analysis provides a numerical estimate of the degree of interaction between solvents and solute and can be a good indicator of their solubility (Hansen, 2007). HSP is based on the assumption that the total energy of cohesion is the sum of the individual contributions of nonpolar interactions (dispersive, related to Van der Waals, D), polar interactions (dipole-dipole and dipole-induced-dipole, P), and hydrogen bonds or other specific associative interactions (including Lewis's acid-base interactions, H). In Eqs. 1–3, to calculate respective solubility parameters, the individual terms for the cohesive energy ($F_{D,i}$, $F_{P,i}$, and $F_{H,i}$) were divided by the molar volumes (V_m):

$$\delta_D = \frac{\sum F_{D,i}}{V_m} \quad \text{(Eq. 1)}$$

$$\delta_P = \frac{\sqrt{\sum F_{P,i}^2}}{V_m} \quad \text{(Eq. 2)}$$

$$\delta_H = \frac{\sqrt{\sum F_{H,i}}}{V_m} \quad \text{(Eq. 3)}$$

The miscibility of phenolic acids in solvents was determined according to the total solubility parameter (Eq. 4).

$$\delta_T^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \quad \text{(Eq. 4)}$$

The HSP of multi-component solvents (δ_{mix}) was calculated from the fraction of the individual components in the solvent mixture (φ_i) and their share in the mixture's HSP (δ_i). The affinity of phenolic acids for multi-component solvents was determined by the distance of the HSPs (R), which is the distance between the points representing phenolic acids (solute) ($\delta_{D,A}$, $\delta_{P,A}$, $\delta_{H,A}$) and the solvent ($\delta_{D,B}$, $\delta_{P,B}$, $\delta_{H,B}$) (Eqs. 5–6).

$$\delta_{mix} = \sum_i \varphi_i \delta_i \quad \text{(Eq. 5)}$$

$$R = \sqrt{4(\delta_{D,A} - \delta_{D,B})^2 + (\delta_{P,A} - \delta_{P,B})^2 + (\delta_{H,A} - \delta_{H,B})^2} \quad \text{(Eq. 6)}$$

A second-order kinetic equation was assumed to describe the kinetics of extraction (Xie et al., 2019) (Eqs. 7–8):

$$C_t = \frac{k \cdot t \cdot C_e^2}{1 + k \cdot t \cdot C_e} \quad \text{(Eq. 7)}$$

$$ER = k \cdot C_e^2 \quad \text{(Eq. 8)}$$

where: C_t – phenolic acid concentration (µg/g DM), t – time of extraction (min), k – rate constant for extraction (g DM/(µg·min)), C_e – equilibrium phenolic acid concentration (µg/g DM), and ER – extraction rate (µg/(g DM·min)).

TFC, TPC, phenolic acid content, ABTS, FRAP, and DPPH were expressed as mean±standard deviation. All extractions were performed in duplicate, and analyses were performed in triplicate. The statistical significance of differences in extraction yields and rates was assessed by ANOVA and post-hoc Duncan's test; the test results are shown in letters in Figs. 1 and 3 and Table 1. Principal Component Analysis (PCA), based on the matrix correlation, was used to determine the relationships between the TPC, TFC, phenolic acid content, antioxidant activity, solvent concentration, and extraction parameters. The PCA results were visualized using a correlogram with RStudio Version 1.2.1335. For statistical analyses, STATISTICA 13.1 (StatSoft) was used, with $p \leq 0.05$ defined as significant.

3. Results and Discussion

3.1. TPC and TFC

In the present study, optimization of polyphenol recovery from distillery stillage embraced the examination of the solid-to-solvent ratio, solvent composition, and time of CSLE, UAE, and MAE.

The pH of the distillery stillage was 6.52, and in extracts, it ranged from 3.68 to 5.11 for CSLE, from 3.71 to 4.89 for UAE, and from 3.39 to 4.97 for MAE extracts. Motikar et al. (2020) showed that the extraction of polyphenols is influenced by the solvent pH, with the best results observed in an acidic environment (pH 4), because at a low pH, phenolic compounds undergo protonation and interact more strongly with the solvent. Using EA as a co-solvent, the TPC and TFC extracted with MAE were the highest, lower values were recorded with UAE, and the lowest values, with CSLE (Fig. 1). The lowest efficiency of CSLE resulted from the fact that polyphenols in the distillery stillage may be bound to the sample matrix (Kumar and Goel, 2019). Ultrasound and microwaves can disrupt the intermolecular interactions between the target compounds and the sample matrix. UAE is based on acoustic cavitation, in which gas bubbles are first formed by ultrasonic waves passing through a liquid and then rupture; bubble implosions cause the release of bioactive compounds (Vinatoru et al., 2017). In MAE, the energy of microwaves increases the solvent diffusivity to the sample (Vinatoru et al., 2017).

In the present study, the microwave power of 400 W and ultrasound frequency of 25 kHz were used, as optimized in the preliminary study (data not shown). To increase the permeability of the cell walls, an ultrasound frequency between 20 kHz and 100 MHz is typically used, depending on the material (Da Porto et al., 2013). Gonzalez-Centeno et al. (2014) reported that a frequency of 40 kHz provided higher yields of grape-derived phenols than one of 120 kHz. When using MAE, Alara et al. (2018) improved the recovery of flavonoids and phenols from leaves with an increase in microwave power from 400 to 500 W; above 500 W the process efficiency decreased. Hence, the microwave power and the ultrasound frequency used to improve the extraction depend primarily on the material from which the polyphenolic compounds will be recovered. In the present study, to increase the extraction efficiency, MAE was performed at 50°C. Although polyphenols are considered

thermolabile, the temperature below 60–80°C should not have caused their decomposition (Antony and Farid, 2022).

3.1.1. Effect of extraction time

Although the extraction yield tends to increase with increasing extraction time, with prolonged exposure to light radiation (CSLE), cavitation (UAE), and microwave radiation (MAE), the risk of degradation of polyphenols increases (Wang et al., 2010). In the present study, extending CSLE time from 15 to 60 min increased the extracted TPC and TFC by about 46% and 41%, respectively, but further extending the time decreased the recovery to 55% (TPC) and 47% (TFC) (Fig. 1ab). At the longest time (90 min), the TFC was similar and the TPC was lower than the values obtained with 15 min of CSLE. Extending the UAE time from 3 to 15 min increased TPC in the extracts by 33% and TFC by 50% (Fig. 1 cd). However, a further extension to 20 min reduced the extraction yield to 46% (TPC) and 48% (TFC); TPC was lower and TFC was similar to the value obtained when UAE lasted 3 min. MAE was not only the most efficient extraction method but the time necessary to reach its highest possible efficiency was shorter than that of the other methods. With an increase in MAE time from 1 to 8 min, the value of TPC and TFC increased by 55% and 59%, respectively. In the most effective MAE and UAE variants, the energy consumption was 53.3 Wh and 141.3 Wh, respectively, which is significant in the economic assessment when selecting the extraction method. In all the extraction techniques, there was a consistent pattern in the way TPC and TFC values changed depending on the extraction time. First, the amount of the compounds that were extracted increased sharply as they were easily extracted from the matrix (15–60 min with CSLE, 3–15 min with UAE, and 1–8 min with MAE). Then, due to interactions between the compounds and the matrix, their recovery became more difficult. Therefore, with the lengthening of CSLE from 60 to 90 minutes, UAE from 15 to 20 minutes,

and MAE from 8 to 10 minutes, the TPC and TFC decreased. This indicates that shorter times were sufficient to extract less strongly bound compounds. For the compounds that were not strongly bound, excessively long extraction times may cause a loss of their chemical stability due to oxidation and degradation (Wang et al., 2010), thus resulting in the decreasing content of polyphenols in the extracts in the present study. Such conditions favor the formation of free radicals, which changes the structure of the polyphenols so that they may no longer exhibit antioxidant activity (Flora, 2009).

3.1.2. Effect of solid-to-solvent ratios

With a solid-to-solvent ratio of 1:30 (w:v), the TPC in the extracts was only 2–4% higher than that obtained with a ratio of 1:15 (w:v); there were no significant effects of this ratio on the extraction yield (Fig. 1). This indicates that the solvent strength was sufficient to obtain similar TPC values with both tested ratios. In the phenolic compound recovery reaction, an equilibrium is reached, after which increasing the solvent amount does not influence the efficiency of recovery (Vinatoru et al., 2017). A low solid-to-solvent ratio may be appropriate when producing a concentrated extract. Therefore, the recovery of polyphenols with a ratio of 1:15 (w:v) can be considered an efficient and, above all, economic process, not only due to the extraction cost but also extract purification.

With a ratio of 1:30 (w:v), about 20% (MAE), 17% (UAE) and 14% (CSLE) higher TFC was obtained than with one of 1:15 (w:v) (Fig. 1a-f). Because flavonoids have a complex structure and often interact with the sample matrix (Alara et al., 2018), large amounts of solvent, up to 1:50 (w:v) should be used to obtain high-quality extracts (Vinatoru et al., 2017).

Special attention should be paid to minimizing the use of the solvent and maximizing the extraction efficiency. Large volumes of solvent require more microwave energy, which is absorbed by the solvent, and ultrasonic waves passing through the liquid, which can

drastically increase the rate of solvent heating, and consequently cause the release of bioactive compounds from the matrix (Alara et al., 2018). Too much heating of the solvent may contribute to the degradation of thermolabile compounds, such as phenolic acids (Zilic et al., 2013). Too little solvent may be insufficient for the recovery of total polyphenols, but small volumes of solvent are favorable to mass transfer because the distribution of the active compounds is concentrated in certain areas, which limits the movement of the compounds out of the sample matrix (Vilkhu et al., 2008).

3.1.3. Effect of the solvent composition

As shown in Fig. 1, the significantly highest recovery yield in terms of TPC was observed with an EA concentration of 70% (EA:EtOH:water of 70:20:10, v:v:v), and in terms of TFC, with an EA concentration of 80% (EA:EtOH:water of 80:10:10, v:v:v). The recovery efficiency at different EA concentrations decreased in the following order: for TPC, 70% > 80% > 60% > 90% > 100% (Fig. 1a, c, e); and for TFC, 80% > 90% > 70% > 60% > 100% (Fig. 1b, d, f). Increasing solvent concentration above 70 and 80% significantly reduced TPC and TFC, respectively (r_s=(-0.87)–(-0.98); $p \leq 0.05$) (Table 1SM).

Bioactive compounds are preferably dissolved in solvents that are similar in polarity to them (Xie et al., 2019). Because polyphenols are polar (phenolic acids have higher polarity than flavonoids) and that water and EtOH have higher polarity than EA, a mixture of water, EtOH, and EA recovered phenolic compounds more effectively than pure EA in the present study. Overall, water improves mass transfer between solid and liquid by increasing the permeability of the matrix (Zhong et al., 2016). This was clearly seen during polyphenol extraction from distillery stillage with 100% EtOH; the TPC was below 2.5 mg GAE/g (MAE) and 2.0 mg GAE/g (UAE) (Mikucka et al., 2022a), similarly to the values obtained in the present study with 100% EA. Adding water to obtain 80% EtOH resulted in more than

twice higher polyphenol yield (Mikucka et al., 2022a). In the present study, each solvent mixture contained the same proportion of water (10%, v:v) but EtOH was added to the solvent mixture because EA is poorly soluble in water but dissolves well in EtOH. The studies on using such a solvent mixture are scarce. With the EA:EtOH:water mixture used to separate polyphenols, all catechins were well resolved when the relative volume of EtOH was small (20:1:20, 25:1:25, 50:1:50, or even 1:0:1, v:v:v) (Cao et al., 2001). When separating phenolic compounds from grape seed, changing the proportion of the solvent in the hexane:EA:EtOH:water mixture (1:8:2:7 and 4:5:3:3, v:v) resulted in extracting different phenolic compounds (Delaunay et al., 2002). In the present study, the use of EA:EtOH:water mixtures resulted in almost twice higher TPC in the extracts than 100% EA or 100% EtOH (Mikucka et al., 2022a). Similar surface tension of all solvent mixtures in the present study (23.26–23.97 mN/m) indicated that it did not affect the recovery efficiency, as described by Wang et al. (2008). Thus, it was EtOH added to the mixture of extraction solvents that had the strongest effect on recovery efficiency. Due to the high dielectric constants of water (ε=78.39) and EtOH (ε=24.55), they absorb energy, which can lead to faster heating of the solvent (Zhong et al., 2016). Moreover, for the extraction of thermolabile compounds, like polyphenols, a combination of solvents with relatively lower dielectric properties, such as EA (ε=6.02), can be used to ensure that the temperature of the solute remains lower when released into the solvent (Routray and Orsat, 2012). Thus, in the present study, as the amount of EtOH in the solvent mixture increased above 20% and that of EA decreased below 70%, there was a corresponding decrease in the amount of extracted compounds (Fig. 1). All these observations show that selecting the appropriate solvent mixture by modifying the polarity and the dielectric constant improves extraction efficiency (Xie et al., 2019), which confirms the need to optimize solvent concentrations for newly tested raw materials.

For the optimal selection of the solvent components, their affinity for the target compounds should be determined. This affinity can be estimated using the HSP (Hansen, 2007). Fig. 2 shows the distances between solubility parameters (R) depending on the composition of the extraction solvent mixture. The R values decreased as the concentration of EA increased, reaching a minimum at 70–80% EA, then they increased, which is related to the contribution of dispersive interactions, polar interactions, and hydrogen bond interactions of solvents and phenolic acids, according to Hansen's solubility theory (Table 2SM). The strength of dispersive interactions of EtOH and EA had the strongest effect on the R-value, mainly due to the presence of the $-CH_3$ and $-CH_2-$ groups, and additionally that of the -COO- group (EA). Additionally, in the case of EtOH and water, the R values were highly dependent on the interactions of hydrogen bonds, due to the presence of -OH groups in these molecules. Moreover, phenolic acids are characterized by both high dispersive interactions and hydrogen-bond interactions due to the presence of an aromatic ring and $-CH_3$ groups, and -COOH and -OH groups, respectively (Hansen, 2007). The interaction forces between the molecules of all components were similar in the present study and indicated that the phenolic compounds were highly soluble. Lower R values indicate greater thermodynamic probabilities that the solute and solvent will dissolve each other. As the lowest R values were obtained with 70% and 80% (v:v) EA, these solutions seem to be the most optimal solvents for extracting phenolic acids (Fig. 2). This similarity in the effects of these two solutions may result from interactions between phenolic acids and other components of raw material that form complex products and from the different affinity of these products for solvents (Xiang and Wu, 2017).

3.2. Phenolic acids

3.2.1. Quantification of phenolic acids

In the HPLC analysis, high-quality peak shapes and good separation were obtained, as well as minimal baseline variability (Fig. 1SM). Both after hydrolysis (extracts containing total phenolic acids) and after extraction without hydrolysis (extracts containing free phenolic acids), the phenolic acid profiles were similar. The average total concentration of phenolic acids was 2.94±0.53 µg/g DM. The concentration of free phenolic acids extracted in CSLE, UAE, and MAE accounted for 69%, 89%, and 95% of their total concentration, respectively. Thus, it can be assumed that bound phenolic acids accounted for a lesser part of the total phenolic acid content in the distillery stillage. Therefore, the phenolic acids that are present in the distillery stillage can be recovered by extraction, skipping the hydrolysis step which uses toxic reagents. This can be explained by the fact that, during the fermentation process, bound phenolic acids are released from the matrix of cereal raw materials (Skrajda-Brdak et al., 2019).

In the present study, the significantly highest recovery of free phenolic acids with a solid-to-solvent ratio of 1:15 (w:v) was obtained with a 70% EA with 8-min MAE: 2.59±0.29 µg/g DM (Fig. 3 c). With UAE, the concentrations of phenolic acids were slightly lower and amounted to 2.46±0.27 µg/g DM during 15-min extraction with 70% EA (Fig. 3 b). The extracts obtained with 60-min CSLE using 70% EA contained 1.97±0.17 µg/g DM (Fig. 3 a). The concentrations of phenolic acids obtained with other variants of CSLE, UAE, and MAE are shown in Fig. 2SM. To the best of our knowledge, no research has been conducted on the recovery of phenolic acids from distillery stillage coming from the alcoholic fermentation of cereals. However, cereals themselves have been extensively tested for phenolic acid content, e.g. 177–257 µg/g DM (Yu et al., 2013) or 867.38 µg/g DM (Zilic et al., 2013) in wheat, or 4.63–7.24 µg/g DM (Skrajda-Brdak et al., 2019) in whole grain flour. Despite the high efficiency of the phenolic acid extraction from distillery stillage in the present study, the content in the extracts was much lower. This is because the fermentation of cereals causes

most of the phenolic acids to pass into the product, while only some part remains in the by-products.

In this study, ferulic and *p*-coumaric acids, which belong to hydroxycinnamic acids, predominated among the recovered phenolic acids, ranging 31–36% and 25–28% of free phenolic acids, respectively (Fig. 3). The shares of vanillic acid (15–17%) and sinapic acid (11–14%) were much smaller. The content of p-OH benzoic and syringic acids was the lowest; together they accounted for about 5–7% of the free phenolic acids. The composition of phenolic acids is related to their chemical structure. Derivatives of hydroxybenzoic acids exist in a bound form, and structural changes occur because of hydroxylation and methylation of the aromatic ring (Wang et al., 2008). Derivatives of hydroxycinnamic acids are characterized by a backbone with a double bond in the side chain (*cis* or *trans*) (Chen et al., 2020). Most ferulic acids and *p*-coumaric acids found in plants are bound to cell wall polysaccharides via ester bonds. Breaking these bonds releases bound phenolic acids, resulting in a higher overall yield of phenolic acids (Nara et al., 2006). This explains why, in the present study, *p*-coumaric and ferulic acids were mainly released and to a lesser extent broken down in fermentation, as may happen with other phenolic acids. Moreover, ferulic acid may be produced by the transformation of vanillic acid (Wang et al., 2008), which may also explain the predominance of ferulic acid in the extracts obtained in the present study.

3.2.2. Extraction kinetics

The extraction rates were calculated for the periods in which an increase in phenolic acid content was observed (0–60 min (CSLE), 0–15 min (UAE), and 0–8 min (MAE)) (Table 1). For all the experimental data, the determination coefficient R^2 was ≥ 0.99, which indicated a good matching between the experimental data and the proposed second-order kinetic model. The maximum extraction rates of total phenolic acids were achieved with 70% EA

(1.824±0.44 and 2.023±0.47 μg/(g DM·min) with MAE, with solid-to-solvent ratios of 1:15 and 1:30 (w:v), respectively) (Table 1). With a solid-to-solvent ratio of 1:30 (w:v) in MAE, the extraction rate when using 80% EA was similar to that with 70% EA. In each extraction variant, the extraction rates obtained with MAE were 3- and 10-times higher than those with UAE and CSLE, respectively. The extraction rate was higher when using MAE than when using UAE because the energy of microwave radiation increases the diffusivity of the solvent to a greater extent than the effect of cavitation (Xie et al., 2019).

Among all tested acids, the extraction rates were the highest for ferulic and *p*-coumaric acids. All phenolic acids, except for *p*-coumaric acid, were extracted at the highest rate with 70% EA. Interestingly, *p*-coumaric acid had the highest extraction rate with 60% EA in each UAE and MAE variant. This could be due to the role of EtOH, which accounted for 30% of this solvent. EtOH increases the solubility of antioxidant compounds by reducing the dielectric constant and thus increasing diffusion (Garcia-Salas et al., 2010). To conclude, these results indicate that kinetic modeling successfully explains the process of extraction of antioxidant compounds from distillery stillage and helps select optimal extraction conditions.

3.3. Antioxidant activity

The assays that were used to determine the antioxidant activity of polyphenols employ different reaction mechanisms based on the radical scavenging activity (ABTS and DPPH) or the reduce ferric iron (Fe^{3+}) to iron (Fe^{2+}) (FRAP). Different mechanisms are based on hydrogen atom transfer (HAT) and single electron transfer (SET) (Awika et al., 2003). With natural antioxidants, these mechanisms occur simultaneously. Because the polyphenol structure is a key determinant of their activity, all these tests were carried out to understand the relationship between the structure and the activity of these compounds and to obtain a complete picture of the properties of the extracted compounds. Fig. 4 shows the antioxidant

activity of extracts from CSLE, UAE, and MAE using 70% EA. Table 3SM shows the antioxidant activity of the remaining extracts. The extracts obtained with 70% EA had higher antioxidant activity than the extracts obtained with the other extraction variants (Fig. 4). Because MAE with 70% EA resulted in the highest content of recovered phenolic acids, it can be assumed that phenolic acids were the compounds that were responsible for the antioxidant activity of the obtained extracts. The antioxidant activity were strongly positively correlated with the TPC, TFC, and phenolic acid content (r_s=(0.71)–(0.98); $p \leq 0.05$) (Table 1 SM). The activities of the extracts obtained with 8-min MAE were the highest, as indicated by ABTS (28.02–29.84 μmol TE/g DM), DPPH (16.85–17.41 μmol TE/g DM), and FRAP (4.45–4.63 μmol $FeSO_4$/g DM). In UAE, the extracts obtained with the use of 15-minute sonication had the highest antioxidant activity, which was slightly lower than that mentioned above for MAE. With CSLE, the extracts' antioxidant activity was the highest after the 60-min process. However, these activities were still 1.6- and 1.3-times higher than the respective activities obtained with UAE and MAE performed for the optimal amount of time and with 100% EA. Because extending the extraction time in MAE, UAE, and CSLE longer than the values mentioned above decreased the efficiency of phenolic acid recovery, it also decreased the antioxidant activity of the obtained extracts.

In the present study, hydroxycinnamic acids comprised up to 72% of total free phenolic acids. Therefore, this group of acids appears to be the one that is most responsible for the antioxidant activity of the distillery stillage extracts. The activity of phenolic acids is influenced by their ability to donate electrons. Chen et al. (2020) reported that hydroxycinnamic acids (-CH=CHCOOH) have greater antioxidant activity than hydroxybenzoic acids (-COOH), although both acids have a carboxylic acid group, which can donate electrons. However, coupling and induction effects cause -COOH to be a strong electron-withdrawing group and -CH=CHCOOH to be a weak one. The weak electron-

withdrawing group can increase the electron cloud density of the benzene ring, decrease the dissociation energy of the phenolic hydroxyl bonds, and then increase the free radical scavenging capacity of hydroxycinnamic acids (Chen et al., 2020).

For hydroxycinnamic or hydroxybenzoic acids, substituents on the benzoic ring are essential for their antioxidant activity. The greater the number of -OCH_3 groups, the higher the antioxidant activity of the acids. The -OCH_3 group stabilizes the interaction of its lone electron pair on the oxygen atom, thus increasing its ability to scavenge free radicals (Ammar et al., 2009). In phenolic acids, the number and position of the phenolic -OH are directly related to their free radical scavenging capacity; phenolic -OH on the benzene ring are electron donor groups and thus reduce the dissociation energy of the hydroxyl bonds, which improves their ability to scavenge free radicals (Chen et al., 2020). The antioxidant activity of phenolic acids is increased by the addition of -OCH_3 substituents or a second -OH at the *ortho* position, which allows intramolecular hydrogen bonding. However, the substitution of a 4-position -OH between -OCH_3 groups, as in syringic and sinapic acids, reduces this activity (Shahidi and Zhong, 2015). In general, both phenolic -OH and -OCH_3 influence antioxidant activity. According to the above information, the antioxidant activity of phenolic acids recovered in the present study could decrease in the order: sinapic > ferulic > *p*-coumaric for the hydroxycinnamic acids, and syringic > vanillic > p-OH benzoic for the hydroxycinnamic acids.

In all extracts obtained in the present study, the ABTS assay indicated much higher antioxidant activity values than the DPPH assay, while the FRAP assay indicated the lowest values, although ABTS, DPPH, and FRAP values were strongly positively correlated (r_s=(0.97)–(0.98); $p \leq 0.05$) (Table 1 SM). The data on the ability to simultaneously donate hydrogen atoms and electrons obtained by these methods provide important information regarding the antioxidant activity of polyphenols. For example, Ramirez-Garcia et al. (2021)

found a strong correlation between ABTS and FRAP for cereal samples, but between ABTS and DPPH the correlation was weaker. They found that the FRAP assay correlated well with both the ABTS and DPPH assays. Aouadi et al. (2021) found that the FRAP test correlated negatively with ABTS and DPPH, while ABTS and DPPH correlated positively in medicinal and aromatic plants. These contradictory results are because the dominant antioxidant mechanism in each system depends on the material from which the antioxidants are recovered, their structure and properties, as well as the solvent (Awika et al., 2003). ABTS and FRAP react in an aqueous system, while the DPPH reacts in an EtOH system. According to Cheng et al. (2007), an EtOH system promotes the scavenging of free radicals in the DPPH assay by ferulic and *p*-coumaric acids. However, the antioxidant activity of each phenolic compound is also affected by intermolecular interactions, which can be synergistic or antagonistic, depending on the conditions and the compounds tested (Ammar et al., 2009). Farhoosh et al. (2016) reported that the EtOH system may also affect the intramolecular hydrogen bonds between the -OH and -OCH_3 groups of phenolic acids, and consequently lower their antioxidant activity in the DPPH assay compared to what is observed in the ABTS and FRAP assays.

3.4. PCA

To summarize all the extraction results and to minimize the number of explanatory variables, the data were visualized with PCA. By combining highly correlated variables, the variables could be reduced to two main components, which together accounted for 80.8–84.2% of the total variability (Fig. 5). Highly related variables are positively correlated with one component, while in relation to other components they show a negative correlation. PC1 accounted for 71.9–74.3%, while PC2 accounted for 8.9–9.9% of the variance.

The score plots (Fig. 5 a,c,e) contains all tested coded samples (shown in Table 4SM), while the loadings plots shows the contribution of the dependent variables (TFC, TPC, DPPH, ABTS, FRAP and individual phenolic acids) (Fig. 5 b,d,f). The position of each variable on the plots describes its relationship with other variables. In all extraction methods, TPC, ABTS, FRAP, DPPH and all individual phenolic acids were positively correlated to the PC1 axis. TFC was positively correlated to the PC2 axis, while TPC, ABTS, FRAP, DPPH and all individual phenolic acids were negatively correlated with it. Similar relationships were reported by Ahmadian-Kouchaksaraie et al. (2016) in the subcritical water extraction of phenolic antioxidants from *Crocus sativus* petals of saffron industry residues. The TPC, ABTS, DPPH, FRAP and the content of individual phenolic acids were the highest with CSLE at 60 min and 70% EA ($C_1A_{-1}B_0$, $C_1A_{-1}B_1$; Fig. 5 a,b), with UAE at 15 min and 70% EA ($U_1A_{-1}B_1$, $U_1A_{-1}B_0$; Fig. 5 c,d), and with MAE at 8 min and 70% EA ($M_1A_{-1}B_1$, $M1A_{-1}B_0$; Fig. 5 e,f). This suggests that the antioxidant activity of the extracts was directly influenced by the content of individual phenolic acids and TPC, which depended on the extraction conditions. Synergistic and antagonistic antioxidant interactions can explain why the samples with similar concentrations of total phenolic compounds displayed different antioxidant activities in the different assays. TFC was the highest in the extracts obtained with CSLE at 60 min and 80% EA ($C_1A_0B_1$, $C_1A_0B_0$; Fig. 5 a,b), with UAE at 15 min and 80% EA ($U_1A_0B_1$, $U_1A_0B_0$; Fig. 5 c,d), with MAE at 8 min and 80% EA ($M_1A_0B_1$, $M_1A_0B_0$; Fig. 5 e,f). The results depicted by PCA are consistent with the results discussed above.

4. Conclusions

Distillery stillage is a source of polyphenols that can be recovered *via* ultrasound-assisted, microwave-assisted and conventional extractions with solvent mixtures containing ethyl acetate. The yield and rate of extractions and the antioxidant activity of the extracts depended

on the extraction method, concentration of solvent and time of extraction. Free phenolic acids were mainly extracted, which allows hydrolysis omission, thus avoiding the unnecessary use of toxic reagents. Hydroxycinnamic acids accounted for about 72% of total free phenolic acids extracted, which may decide the future utilization of the extracts in terms of their antioxidant activity. This study can form the basis for further pilot trials of "green extractions" (ultrasound- and microwave-assisted) for recovering phenolic acids, which can contribute to agri-food waste valorization.

Author Contributions

WM: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft Preparation, Visualization. MZ: Conceptualization, Methodology, Validation, Formal Analysis, Writing – Original Draft Preparation, Writing – Review and Editing, Visualization, Supervision, Funding Acquisition. KB: Methodology, Validation, Formal Analysis, Writing – Review and Editing, Supervision. IW: Writing – Review and Editing, Supervision.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1. Total polyphenol content (TPC) and total flavonoid content (TFC) obtained with conventional solid-liquid extraction (CSLE) (a,b), ultrasound assisted extraction (UAE) (c,d), and microwave-assisted extraction (MAE) (e,f). In the abbreviations, the values after CSLE, UAE, and MAE show the time of extraction; the values after EA (ethyl acetate) indicate the concentration of the solvent; the solid-to-solvent ratio is given; the letters indicate the ANOVA results

Fig. 2. Distance of the Hansen solubility parameters (R) for phenolic acids depending on the concentration of ethyl acetate (EA)

Fig. 3. Concentrations (C) of free phenolic acids recovered during conventional solid-liquid extraction (CSLE) (a), ultrasound assisted extraction (UAE) (b), and microwave-assisted extraction (MAE) (c) with a solid-to-solvent ratio of 1:15 (w:v). In the abbreviations, the values after CSLE, UAE, and MAE show the time of extraction; the values after EA (ethyl acetate) indicate the concentration of the solvent; the letters indicate the ANOVA results

Fig. 4. Antioxidant activity of distillery stillage extracts obtained with 70% ethyl acetate (EA) *via* conventional solid-liquid extraction (CSLE) (a), ultrasound assisted extraction (UAE) (b), and microwave-assisted extraction (MAE) (c). In the abbreviations, the values after CSLE, UAE, and MAE show the time of extraction; the solid-to-solvent ratio is given

Fig. 5. Principal Component Analysis (PCA) between total polyphenol content (TPC), total flavonoid content (TFC), the antioxidant activity (DPPH, ABTS, FRAP), and the extractability of individual phenolic acids from distillery stillage by using conventional solid-liquid extraction (CSLE (a,b)), ultrasound-assisted extraction (UAE (c,d)), and microwave-assisted extraction (MAE (e,f)). The score plot of the PCA (a,c,e) is based on the independent variables (time of extraction, concentration of ethyl acetate, solid-to-solvent ratio) depending on the extraction method. The codes of the samples are shown in Table 4SM. The loading plot

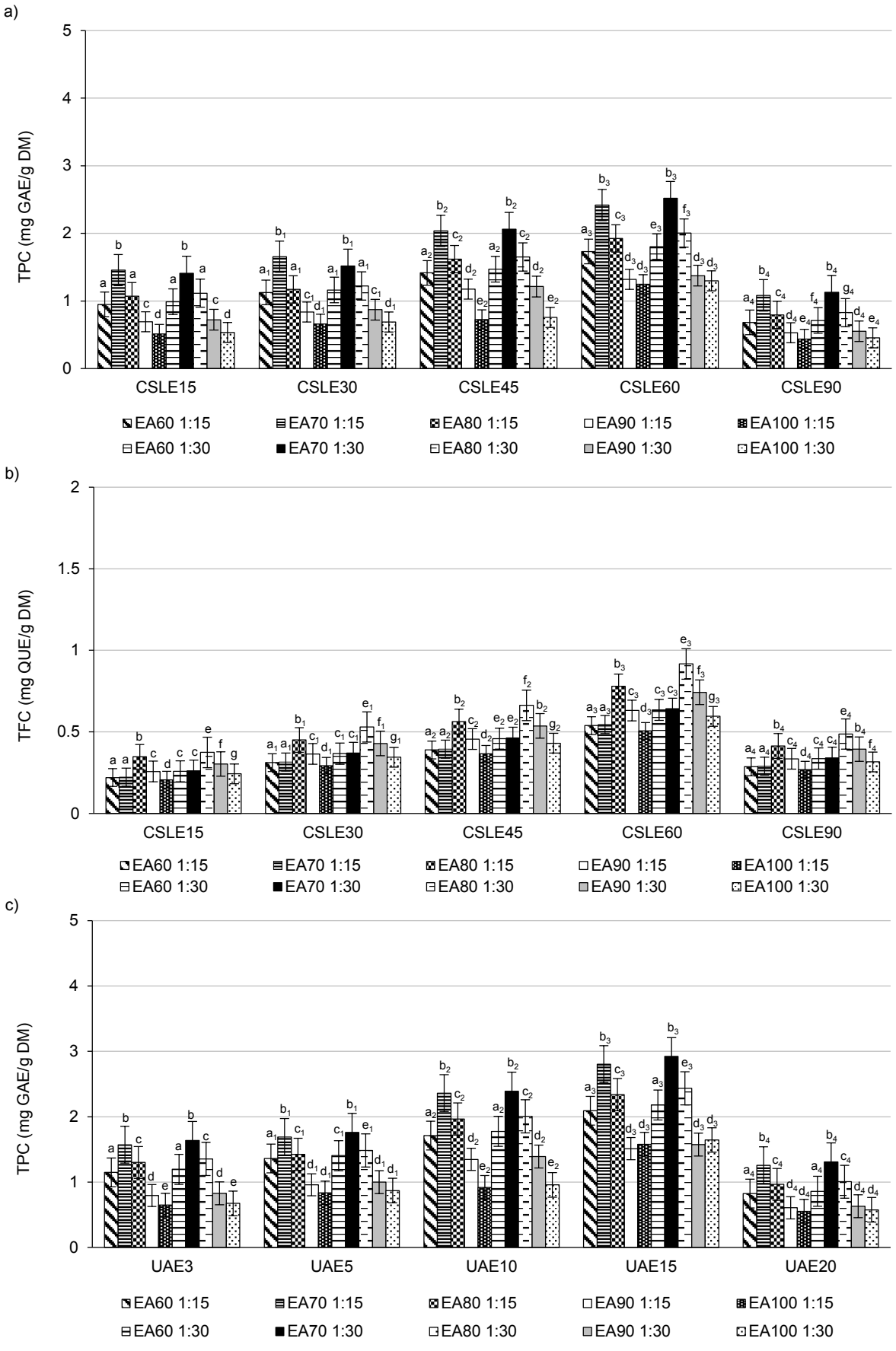
of PCA (b,d,f) represents the contribution of dependent variables (TFC, TPC, DPPH, ABTS, FRAP, and individual phenolic acids); pOH – p-OH benzoic acid, VAN – vanillic acid, SYR – syringic acid, pCO – *p*-coumaric acid, FER – ferulic acid, SIN – sinapic acid

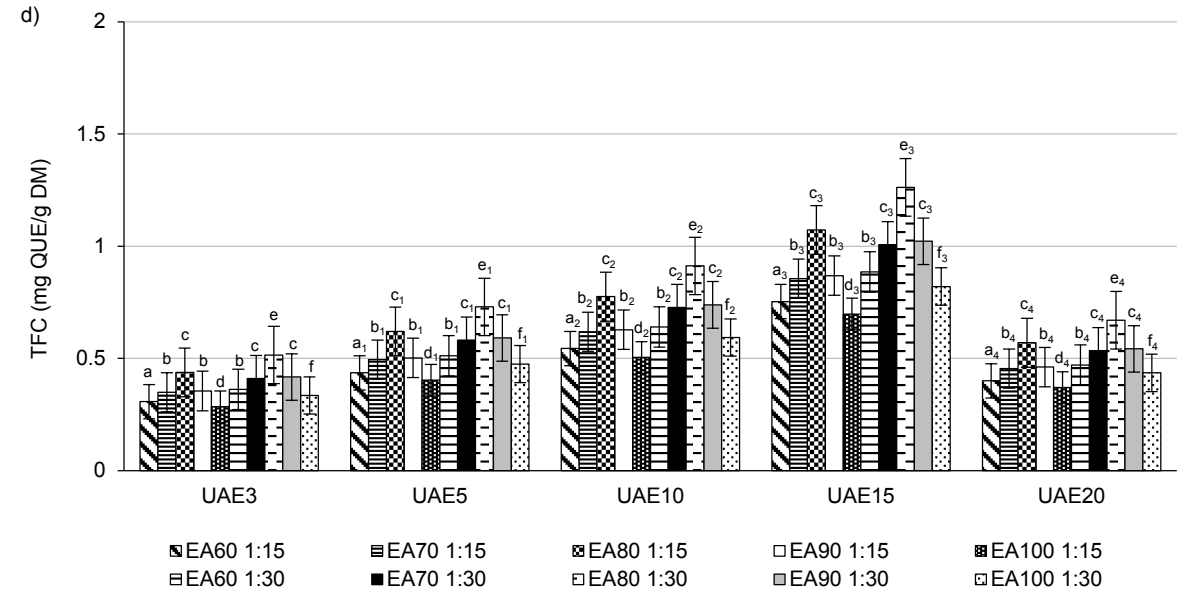
Table 1. Rates of conventional solid-liquid extraction (CSLE), ultrasound assisted extraction (UAE), and microwave-assisted extraction (MAE) of individual free phenolic acids and total free phenolic acids. In the abbreviations, the values after EA (ethyl acetate) indicate the solvent concentration; the solid-to-solvent ratio is given; the letters indicate the ANOVA results

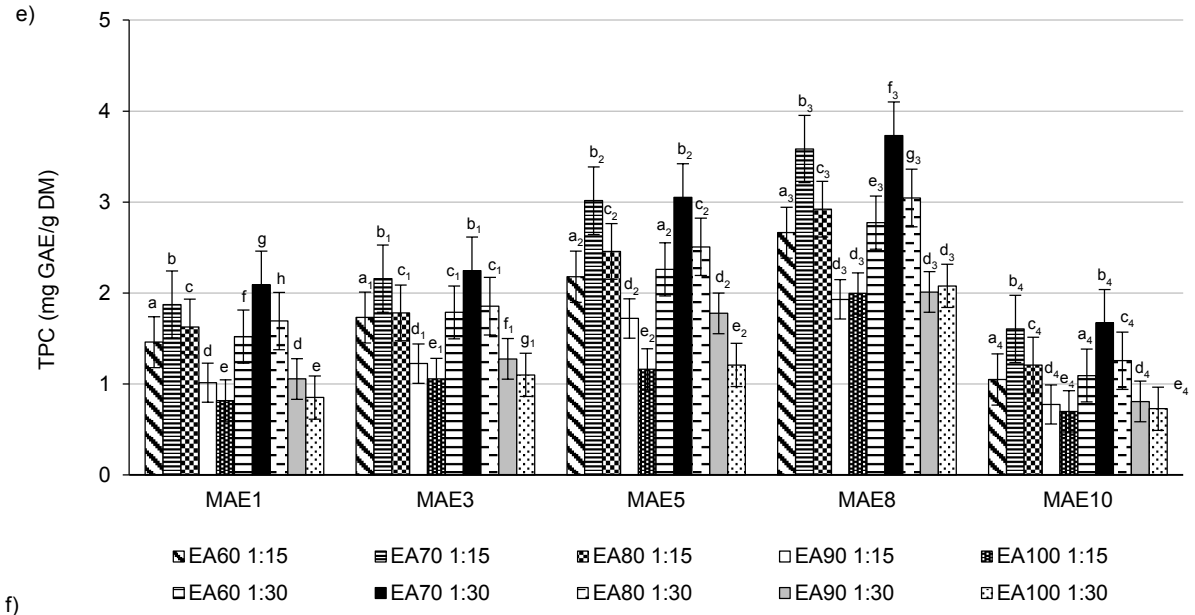
Type of extraction	Extraction rate (µg/(g DM·min))						
	p-OH benzoic	vanillic	syringic	*p*-coumaric	ferulic	sinapic	Total acids
CSLE EA60 1:15	0.002^{a}	0.005^{a1}	0.002^{a2}	0.021^{a3}	0.011^{a4}	0.003^{a5}	0.080^{a6}
CSLE EA70 1:15	0.006^{a}	0.010^{a1}	0.005^{a2}	0.047^{b3}	0.038^{b4}	0.008^{a5}	0.179^{b6}
CSLE EA80 1:15	0.007^{a}	0.012^{a1}	0.005^{a2}	0.025^{c3}	0.046^{c4}	0.008^{a5}	0.133^{c6}
CSLE EA90 1:15	0.004^{a}	0.006^{a1}	0.003^{a2}	0.015^{a3}	0.028^{b4}	0.004^{a5}	0.081^{a6}
CSLE EA100 1:15	0.003^{a}	0.005^{a1}	0.002^{a2}	0.011^{a3}	0.021^{b4}	0.003^{a5}	0.056^{a6}
CSLE EA60 1:30	0.003^{a}	0.005^{a1}	0.003^{a2}	0.019^{a3}	0.011^{a4}	0.004^{a5}	0.085^{a6}
CSLE EA70 1:30	0.007^{a}	0.012^{a1}	0.006^{a2}	0.048^{b3}	0.043^{d4}	0.010^{a5}	0.194^{b6}
CSLE EA80 1:30	0.007^{a}	0.012^{a1}	0.005^{a2}	0.016^{a3}	0.046^{c4}	0.009^{a5}	0.128^{c6}
CSLE EA90 1:30	0.005^{a}	0.007^{a1}	0.003^{a2}	0.010^{a3}	0.012^{a4}	0.005^{a5}	0.080^{a6}
CSLE EA100 1:30	0.003^{a}	0.005^{a1}	0.002^{a2}	0.006^{a3}	0.010^{a4}	0.004^{a5}	0.054^{a6}
UAE EA60 1:15	0.024^{b}	0.044^{b1}	0.020^{b2}	0.187^{d3}	0.108^{e4}	0.032^{b5}	0.385^{d6}
UAE EA70 1:15	0.039^{c}	0.073^{c1}	0.033^{c2}	0.152^{e3}	0.264^{f4}	0.055^{c5}	0.606^{e6}
UAE EA80 1:15	0.052^{d}	0.095^{d1}	0.043^{d2}	0.199^{f3}	0.179^{g4}	0.062^{d5}	0.590^{f6}
UAE EA90 1:15	0.040^{c}	0.061^{e1}	0.027^{e2}	0.142^{g3}	0.199^{g4}	0.038^{e5}	0.403^{g6}
UAE EA100 1:15	0.030^{e}	0.055^{e1}	0.025^{e2}	0.109^{h3}	0.102^{h4}	0.034^{e5}	0.320^{h6}
UAE EA60 1:30	0.030^{f}	0.051^{f1}	0.025^{f2}	0.173^{i3}	0.113^{h4}	0.040^{f5}	0.416^{g6}
UAE EA70 1:30	0.049^{g}	0.084^{g1}	0.039^{d2}	0.149^{e3}	0.289^{i4}	0.072^{g5}	0.668^{i6}
UAE EA80 1:30	0.057^{h}	0.095^{d1}	0.043^{d2}	0.133^{g3}	0.194^{g4}	0.069^{g5}	0.574^{f6}
UAE EA90 1:30	0.044^{i}	0.065^{h1}	0.030^{g2}	0.095^{h3}	0.199^{g4}	0.047^{h5}	0.397^{d6}
UAE EA100 1:30	0.031^{e}	0.050^{f1}	0.022^{h2}	0.068^{j3}	0.107^{e4}	0.036^{e5}	0.299^{j6}
MAE EA60 1:15	0.081^{j}	0.151^{i1}	0.067^{i2}	0.758^{k3}	0.361^{j4}	0.110^{i5}	1.396^{k6}
MAE EA70 1:15	0.109^{k}	0.202^{j1}	0.090^{j2}	0.442^{l3}	0.899^{k4}	0.146^{j5}	1.824^{l6}
MAE EA80 1:15	0.079^{j}	0.146^{i1}	0.067^{i2}	0.305^{m3}	0.658^{l4}	0.106^{i5}	1.333^{m6}
MAE EA90 1:15	0.134^{l}	0.247^{k1}	0.113^{k2}	0.519^{n3}	0.391^{m4}	0.160^{k5}	1.467^{n6}
MAE EA100 1:15	0.100^{m}	0.183^{l1}	0.085^{l2}	0.387^{o3}	0.310^{n4}	0.105^{i5}	0.180^{b6}
MAE EA60 1:30	0.106^{n}	0.175^{l1}	0.088^{m2}	0.668^{p3}	0.378^{m4}	0.142^{j5}	1.491^{o6}
MAE EA70 1:30	0.141^{o}	0.262^{m1}	0.117^{k2}	0.573^{q3}	0.745^{o4}	0.190^{l5}	2.023^{p6}
MAE EA80 1:30	0.105^{n}	0.155^{i1}	0.082^{n2}	0.318^{m3}	0.882^{k4}	0.139^{j5}	2.045^{p6}
MAE EA90 1:30	0.152^{p}	0.237^{n1}	0.109^{o2}	0.314^{m3}	0.440^{p4}	0.173^{m5}	1.397^{k6}
MAE EA100 1:30	0.113^{q}	0.175^{l1}	0.081^{n2}	0.238^{n3}	0.345^{q4}	0.127^{n5}	1.022^{q6}

Figure 1

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d)
TFC (mg QUE/g DM)
UAE3
UAE5
UAE10
UAE15
UAE20
EA60 1:15
EA70 1:15
EA80 1:15
EA90 1:15
EA100 1:15
EA60 1:30
EA70 1:30
EA80 1:30
EA90 1:30
EA100 1:30


e)
TPC (mg GAE/g DM)
MAE1
MAE3
MAE5
MAE8
MAE10
EA60 1:15
EA70 1:15
EA80 1:15
EA90 1:15
EA100 1:15
EA60 1:30
EA70 1:30
EA80 1:30
EA90 1:30
EA100 1:30

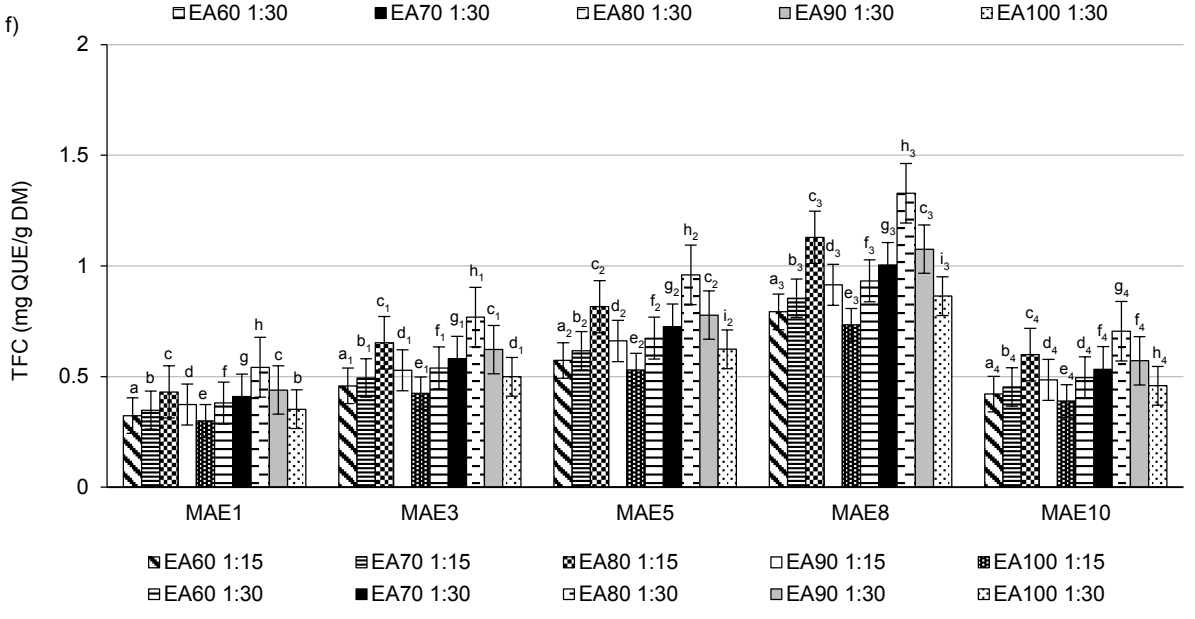

f)
TFC (mg QUE/g DM)
MAE1
MAE3
MAE5
MAE8
MAE10
EA60 1:15
EA70 1:15
EA80 1:15
EA90 1:15
EA100 1:15
EA60 1:30
EA70 1:30
EA80 1:30
EA90 1:30
EA100 1:30

Figure 2

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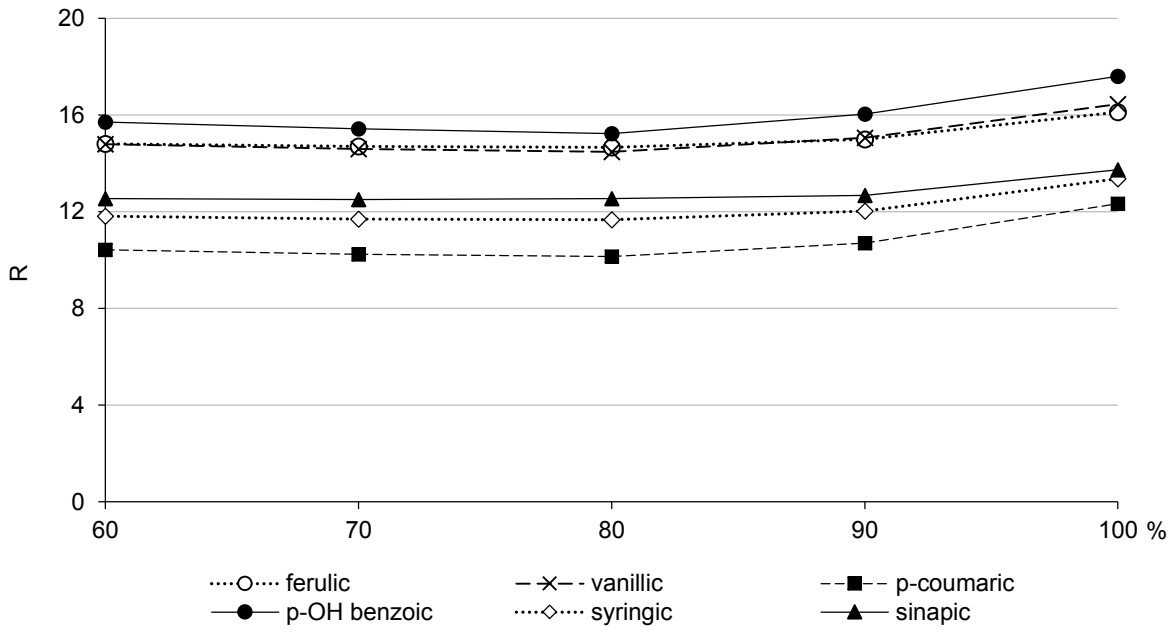


Figure 3

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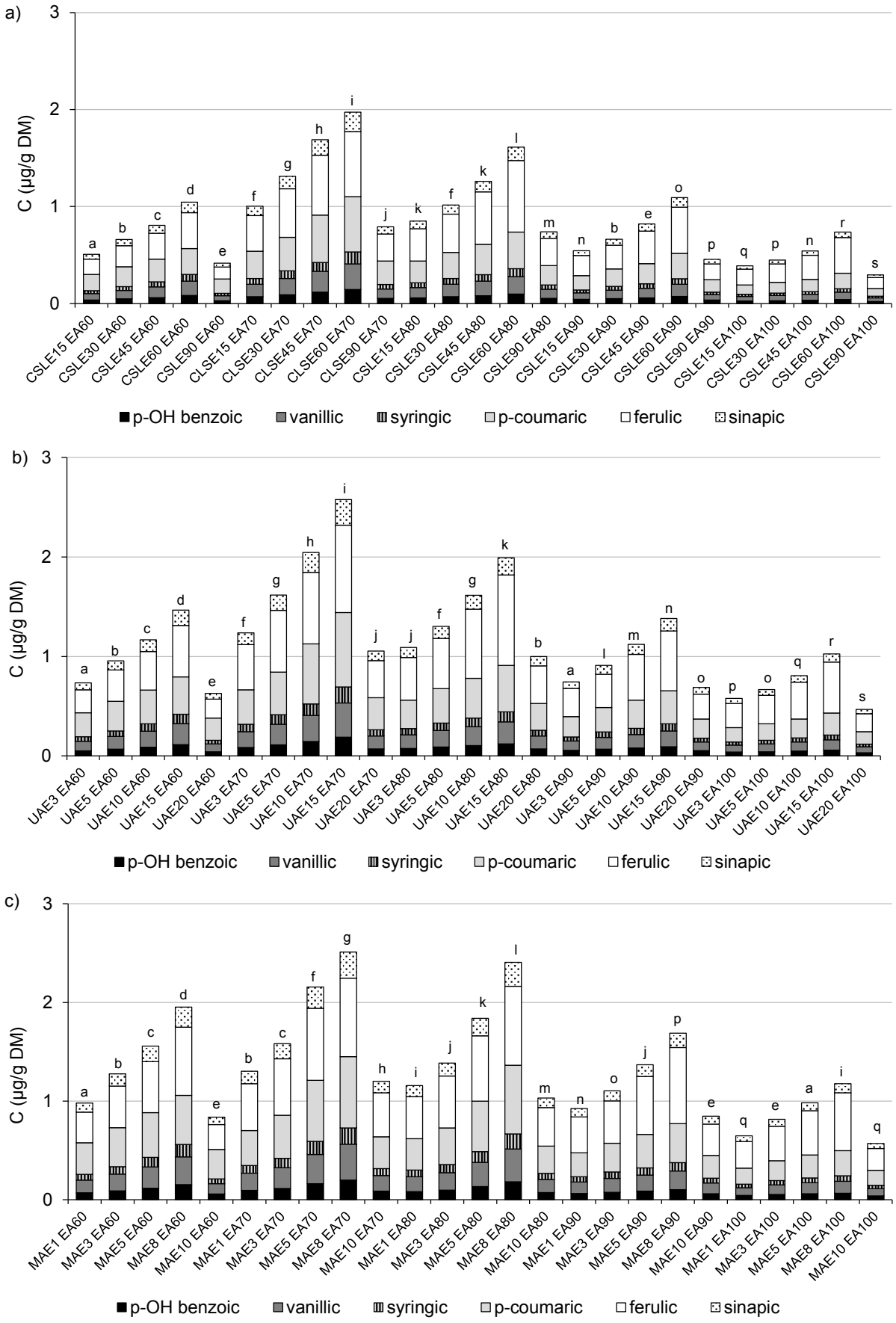


Figure 4

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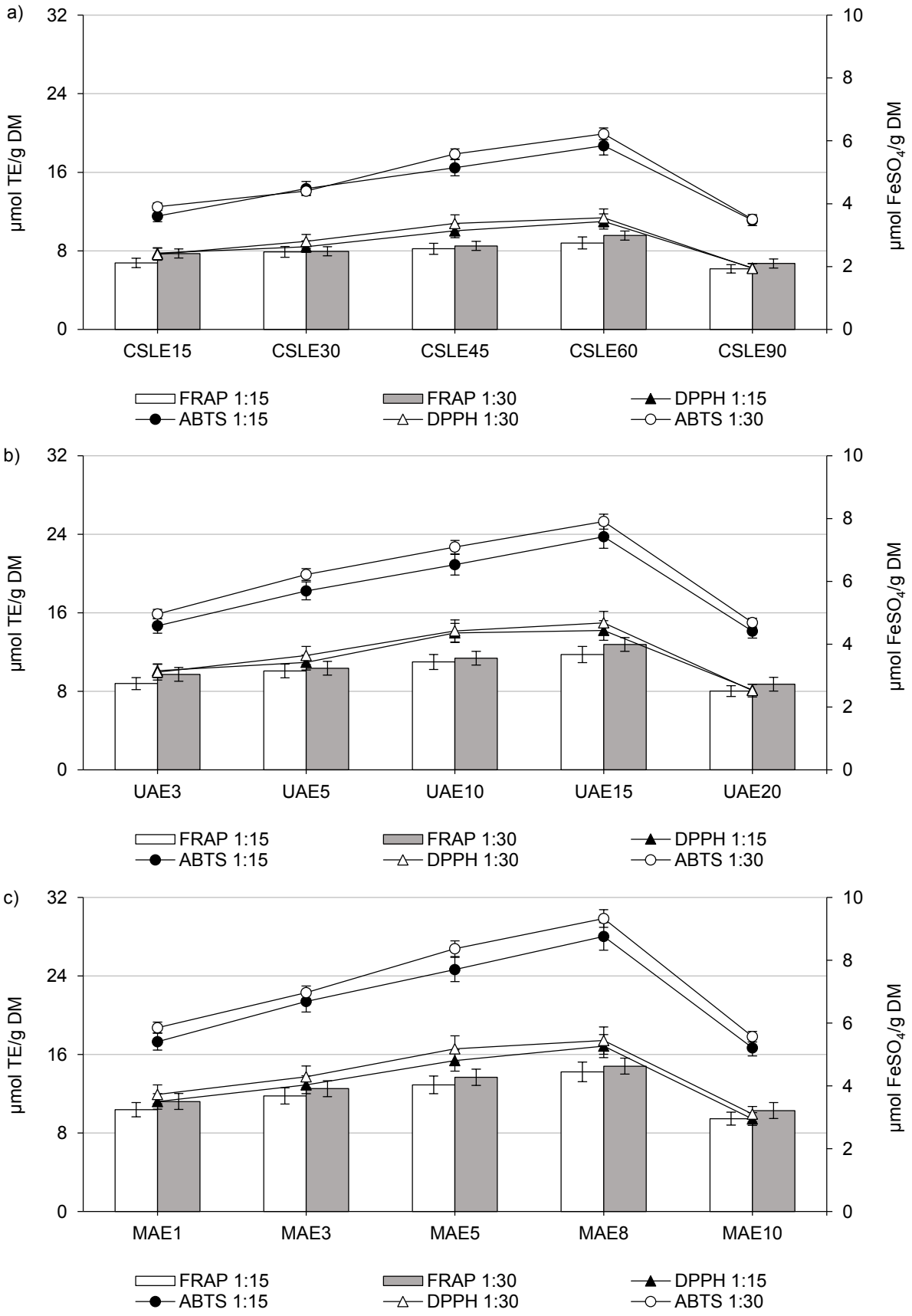
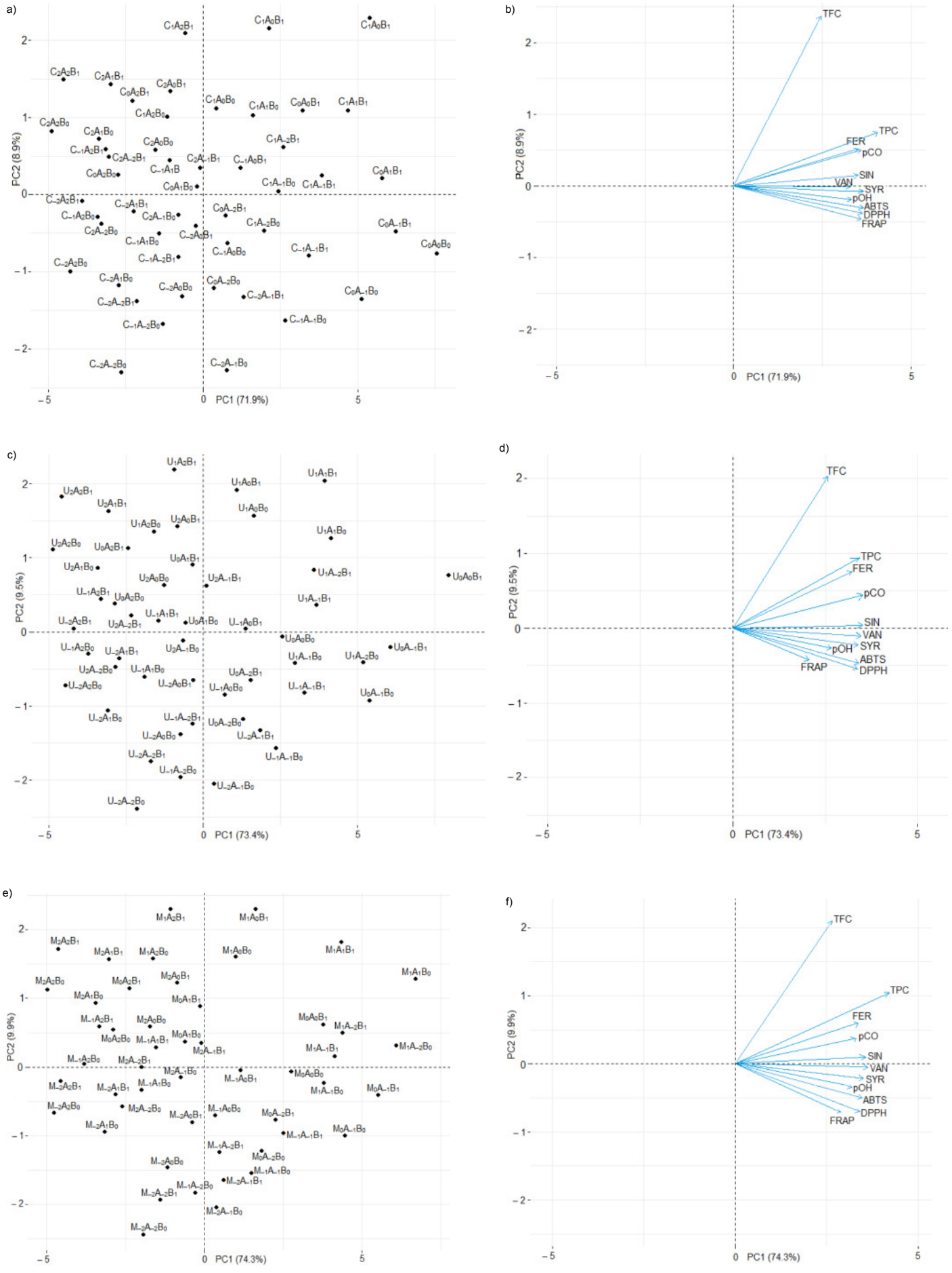


Figure 5

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Valorization of distillery stillage by polyphenol recovery using microwave-assisted, ultrasound-assisted and conventional extractions

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Supplementary Material

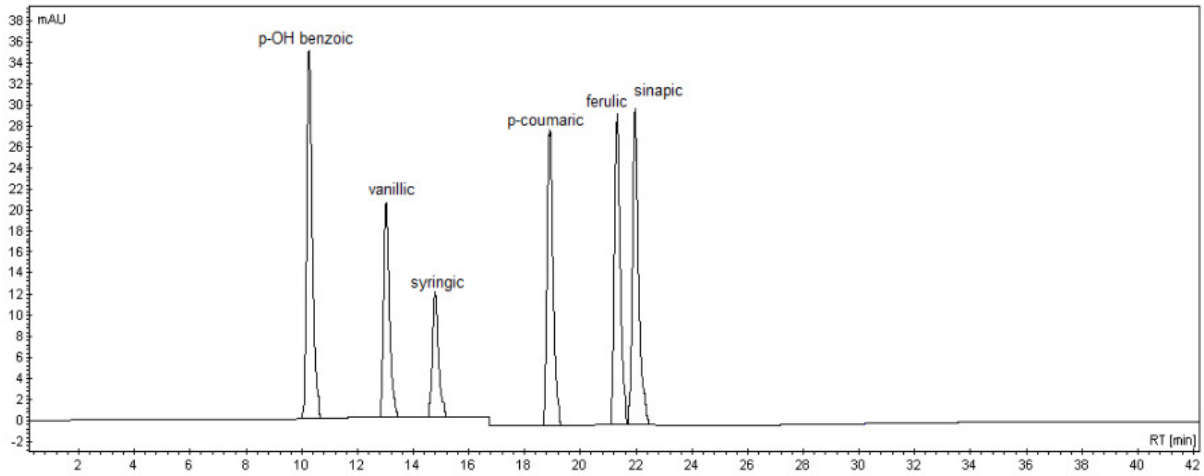


Figure 1SM. HPLC chromatogram of phenolic acid standards at 260 nm (p-OH benzoic, vanillic and syringic acid) and at 320 nm (*p*-coumaric, ferulic and sinapic acid)

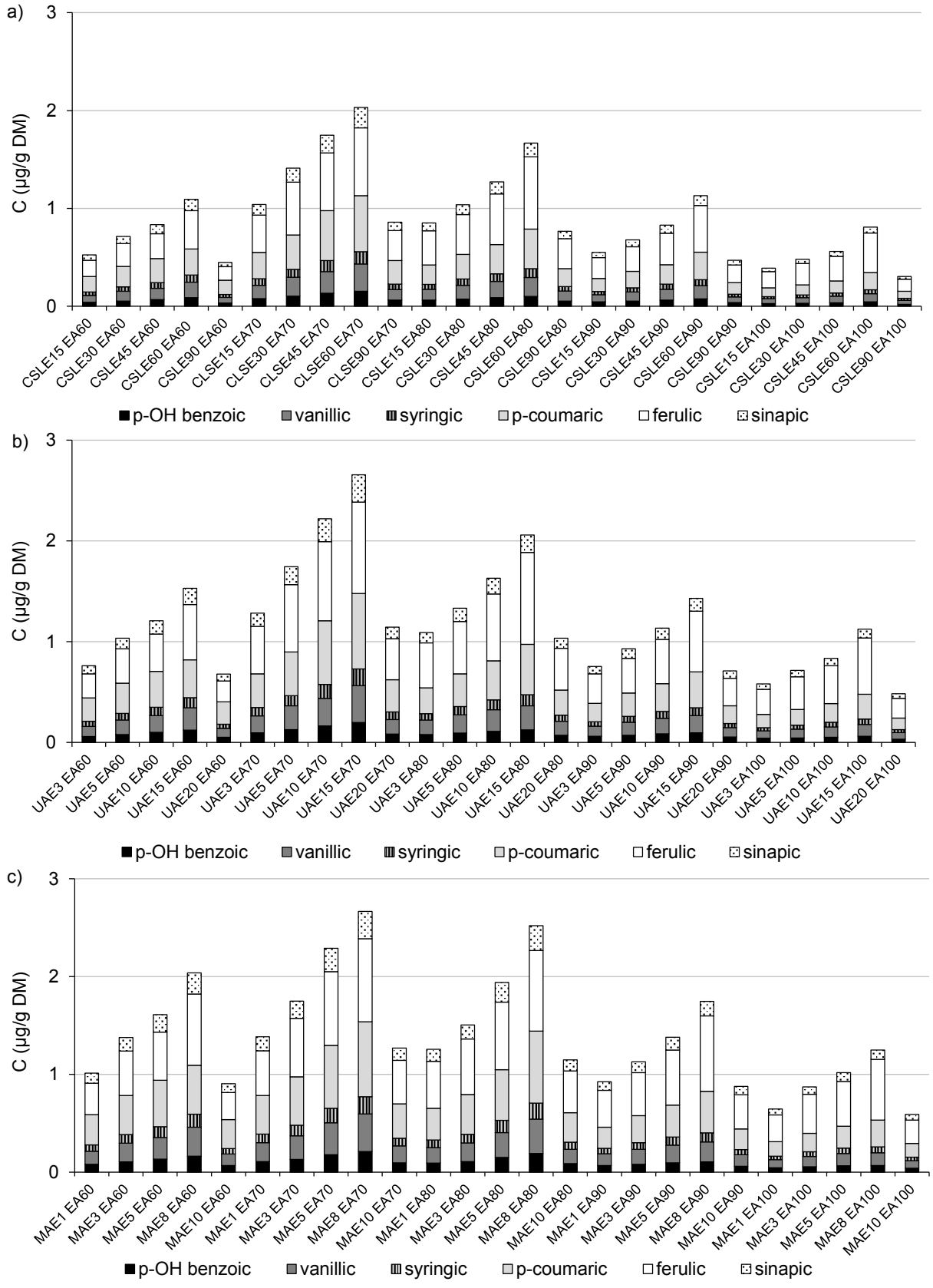


Figure 2SM. Concentrations (C) of free phenolic acids recovered during conventional solid-liquid extraction (CSLE) (a), ultrasound-assisted extraction (UAE) (b), and microwave-assisted extraction (MAE (c) with a solid-to-solvent ratio of 1:30 (w:v). In the abbreviations, the values after CSLE, UAE, and MAE indicate the extraction time; the values after ethyl acetate (EA) indicate the solvent concentration

Table 1SM. The matrix of correlation (r_s) between the Total Polyphenol Content (TPC), Total Flavonoid Content (TFC), phenolic acid concentrations, antioxidant activities (DPPH, FRAP, ABTS), the solvents concentration, and the extraction parameters. Correlation coefficients that are significant with $p \leq 0.05$ are marked with red; r – solid-to-solvent ratio; t – extraction time; C – ethyl acetate concentration

	r	t	C	TPC	TFC	p-OH benzoic	vanillic	syringic	*p*-coumaric	ferulic	sinapic	DPPH	FRAP	ABTS
r														
t	0.00													
C	-0.02	-0.05												
TPC	0.04	-0.29	-0.89											
TFC	0.02	-0.27	-0.87	0.72										
p-OH benzoic	0.08	-0.37	-0.91	0.95	0.71									
vanillic	0.08	-0.37	-0.90	0.96	0.72	1.00								
syringic	0.10	-0.37	-0.92	0.95	0.71	1.00	1.00							
p-coumaric	0.01	-0.36	-0.95	0.95	0.68	0.98	0.98	0.98						
ferulic	0.04	-0.36	-0.98	0.90	0.83	0.89	0.90	0.90	0.88					
sinapic	0.09	-0.36	-0.91	0.95	0.72	1.00	1.00	1.00	0.98	0.91				
DPPH	0.06	-0.60	-0.98	0.95	0.71	0.96	0.95	0.95	0.95	0.98	0.95			
FRAP	0.10	-0.56	-0.96	0.91	0.66	0.93	0.93	0.93	0.92	0.93	0.93	0.97		
ABTS	0.09	-0.69	-0.98	0.96	0.76	0.96	0.96	0.96	0.95	0.99	0.96	0.98	0.97	

Table 2SM. Contribution of dispersive interactions (δ_D), polar interactions (δ_P), and hydrogen bond interactions (δ_H) of solvents and phenolic acids according to Hansen's solubility theory; molar volume (V_m); total solubility parameter (δ_T)

Compound	V_m (mL/mol)	δ_D	δ_P	δ_H	δ_T
water	18.06	15.5	16	42.3	47.81
ethanol	58.39	15.4	8.6	18.5	25.56
ethyl acetate	97.68	15.4	5	8.8	18.43
ferulic acid	136.75	22.7	5.7	15.6	28.13
vanillic acid	119.25	22.6	6.5	16.6	28.78
p-coumaric acid	118.10	20.4	5.6	16	26.52
p-OH benzoic acid	94.60	22.9	7	17.8	29.84
syringic acid	151.28	21.2	5.8	15.4	26.84
sinapic acid	167.32	21.6	5.2	14.7	26.64

Table 3SM. Antioxidant activities of distillery stillage extracts obtained with 60, 80, 90 and 100% ethyl acetate (EA) *via* conventional solid-liquid extraction (CSLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). In the abbreviation, the values after CSLE, UAE, and MAE indicate the extraction time; values after EA indicate the solvent concentration; the solid-to-solvent ratio is given

Type of extraction	Antioxidant activity			Type of extraction	Antioxidant activity			Type of extraction	Antioxidant activity		
	DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)	ABTS (μmol TE/g DM)		DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)	ABTS (μmol TE/g DM)		DPPH (μmol TE/g DM)	FRAP (μmol $FeSO_4$/g DM)	ABTS (μmol TE/g DM)
CSLE15 EA60 1:15	5.60±0.89	1.58±0.44	8.62±0.96	UAE3 EA60 1:15	7.99±0.88	2.18±0.44	11.62±0.79	MAE1 EA60 1:15	8.98±0.95	2.60±0.51	13.86±1.21
CSLE15 EA80 1:15	6.27±0.94	1.92±0.57	10.51±1.11	UAE3 EA80 1:15	8.99±0.94	2.44±0.49	13.05±0.83	MAE1 EA80 1:15	10.09±0.99	2.92±0.57	15.57±1.32
CSLE15 EA90 1:15	5.28±0.95	1.81±0.55	9.05±0.88	UAE3 EA90 1:15	6.56±0.68	1.78±0.33	9.53±0.77	MAE1 EA90 1:15	7.19±0.81	2.08±0.44	11.09±1.06
CSLE15 EA100 1:15	4.19±0.73	1.41±0.53	7.42±0.93	UAE3 EA100 1:15	5.05±0.59	1.37±0.29	7.33±0.68	MAE1 EA100 1:15	5.60±0.55	1.62±0.38	8.65±0.88
CSLE30 EA60 1:15	6.07±0.86	1.84±0.64	10.71±1.08	UAE5 EA60 1:15	8.67±0.63	2.49±0.57	14.44±0.93	MAE3 EA60 1:15	10.34±0.73	2.95±0.56	17.15±1.41
CSLE30 EA80 1:15	6.79±0.84	2.25±0.78	13.07±1.21	UAE5 EA80 1:15	9.74±0.74	2.79±0.62	16.22±1.06	MAE3 EA80 1:15	11.62±0.84	3.31±0.62	19.26±1.52
CSLE30 EA90 1:15	5.72±0.79	2.12±0.72	11.24±1.28	UAE5 EA90 1:15	7.11±0.66	2.04±0.51	11.84±0.83	MAE3 EA90 1:15	8.27±0.86	2.36±0.55	13.72±1.62
CSLE30 EA100 1:15	4.83±0.66	1.64±0.58	8.75±0.96	UAE5 EA100 1:15	5.47±0.57	1.57±0.49	9.12±0.73	MAE3 EA100 1:15	6.45±0.42	1.84±0.32	10.69±1.09
CSLE45 EA60 1:15	7.24±0.82	1.91±0.49	12.28±1.24	UAE10 EA60 1:15	11.04±0.76	2.72±0.55	16.55±1.09	MAE5 EA60 1:15	12.33±1.02	3.23±0.63	19.75±1.66
CSLE45 EA80 1:15	8.10±0.93	2.33±0.82	14.98±1.32	UAE10 EA80 1:15	12.41±0.83	3.06±0.67	18.60±1.16	MAE5 EA80 1:15	13.85±1.09	3.63±0.68	22.19±1.76
CSLE45 EA90 1:15	6.82±0.87	2.20±0.81	12.89±1.35	UAE10 EA90 1:15	9.05±0.54	2.23±0.39	13.57±0.84	MAE5 EA90 1:15	9.86±0.74	2.58±0.55	15.80±1.42
CSLE45 EA100 1:15	5.32±0.61	1.71±0.63	10.57±1.09	UAE10 EA100 1:15	6.97±0.49	1.72±0.28	10.45±0.61	MAE5 EA100 1:15	7.69±0.52	2.02±0.51	12.33±0.99
CSLE60 EA60 1:15	7.92±0.84	2.16±0.83	13.96±1.35	UAE15 EA60 1:15	11.25±0.86	2.97±0.42	18.81±1.21	MAE8 EA60 1:15	13.49±1.02	3.56±0.66	22.45±1.38
CSLE60 EA80 1:15	8.87±0.92	2.64±0.89	17.03±1.66	UAE15 EA80 1:15	12.64±0.91	3.27±0.72	21.13±1.34	MAE8 EA80 1:15	15.16±1.22	4.00±0.38	25.22±1.53
CSLE60 EA90 1:15	7.47±0.63	2.49±0.62	14.65±1.74	UAE15 EA90 1:15	9.22±0.58	2.38±0.61	15.43±0.99	MAE8 EA90 1:15	10.79±0.83	2.85±0.67	17.96±1.38
CSLE60 EA100 1:15	6.29±0.58	1.89±0.51	12.02±1.57	UAE15 EA100 1:15	7.10±0.63	1.84±0.49	11.88±0.87	MAE8 EA100 1:15	8.42±0.68	2.22±0.59	14.01±1.23
CSLE90 EA60 1:15	4.53±0.62	1.44±0.43	8.30±0.99	UAE20 EA60 1:15	6.46±0.59	1.99±0.52	11.19±0.68	MAE10 EA60 1:15	7.55±0.46	2.37±0.49	13.36±1.41
CSLE90 EA80 1:15	5.07±0.68	1.76±0.49	10.13±1.02	UAE20 EA80 1:15	7.26±0.68	2.23±0.66	12.58±0.73	MAE10 EA80 1:15	8.48±0.69	2.66±0.58	15.01±1.32
CSLE90 EA90 1:15	4.27±0.72	1.66±0.52	8.72±0.84	UAE20 EA90 1:15	5.29±0.51	1.63±0.45	9.18±0.59	MAE10 EA90 1:15	6.04±0.56	1.89±0.48	10.68±0.99
CSLE90 EA100 1:15	3.41±0.56	1.29±0.39	6.90±0.75	UAE20 EA100 1:15	4.08±0.48	1.25±0.39	7.07±0.48	MAE10 EA100 1:15	4.71±0.42	1.48±0.44	8.33±0.79

CSLE15 EA60 1:30	5.52±0.63	1.80±0.47	9.33±0.54	UAE3 EA60 1:30	7.88±0.72	2.41±0.69	12.57±0.88	MAE1 EA60 1:30	9.56±0.72	2.81±0.39	15.00±0.99
CSLE15 EA80 1:30	6.18±0.74	2.19±0.53	11.38±0.88	UAE3 EA80 1:30	8.85±0.85	2.70±0.72	14.13±0.94	MAE1 EA80 1:30	10.74±0.81	3.15±0.55	16.86±1.01
CSLE15 EA90 1:30	5.20±0.53	2.07±0.46	9.79±0.94	UAE3 EA90 1:30	6.46±0.67	1.97±0.53	10.31±0.63	MAE1 EA90 1:30	7.65±0.57	2.25±0.62	12.00±1.05
CSLE15 EA100 1:30	4.39±0.48	1.57±0.42	8.03±0.86	UAE3 EA100 1:30	4.98±0.62	1.52±0.47	7.94±0.55	MAE1 EA100 1:30	5.97±0.43	1.75±0.51	9.36±0.78
CSLE30 EA60 1:30	6.46±0.62	1.85±0.38	10.51±0.96	UAE5 EA60 1:30	9.22±0.93	2.56±0.55	15.75±0.89	MAE3 EA60 1:30	11.00±0.87	3.13±0.87	17.87±1.23
CSLE30 EA80 1:30	7.23±0.75	2.26±0.58	12.82±1.28	UAE5 EA80 1:30	10.36±0.77	2.88±0.63	17.70±1.11	MAE3 EA80 1:30	12.36±0.94	3.52±0.77	20.08±1.46
CSLE30 EA90 1:30	6.09±0.69	2.13±0.63	11.04±1.14	UAE5 EA90 1:30	7.56±0.41	2.09±0.46	12.92±0.69	MAE3 EA90 1:30	8.80±0.75	2.51±0.39	14.29±1.21
CSLE30 EA100 1:30	5.13±0.57	1.66±0.49	9.05±1.01	UAE5 EA100 1:30	5.82±0.32	1.62±0.43	9.95±0.53	MAE3 EA100 1:30	6.87±0.53	1.96±0.44	11.15±0.98
CSLE45 EA60 1:30	7.79±0.74	1.98±0.56	13.33±1.21	UAE10 EA60 1:30	11.20±0.55	2.81±0.54	17.97±1.21	MAE5 EA60 1:30	13.27±0.97	3.42±0.67	21.44±1.56
CSLE45 EA80 1:30	8.72±0.82	2.42±0.63	16.26±1.63	UAE10 EA80 1:30	12.59±0.92	3.16±0.74	20.19±1.22	MAE5 EA80 1:30	14.91±1.01	3.85±0.74	24.09±1.62
CSLE45 EA90 1:30	7.35±0.71	2.28±0.65	13.99±1.72	UAE10 EA90 1:30	9.19±0.68	2.31±0.69	14.73±0.75	MAE5 EA90 1:30	10.62±0.83	2.74±0.71	17.15±1.28
CSLE45 EA100 1:30	5.69±0.58	1.77±0.50	11.18±1.43	UAE10 EA100 1:30	7.07±0.72	1.78±0.49	11.35±0.63	MAE5 EA100 1:30	8.28±0.69	2.14±0.52	13.38±1.11
CSLE60 EA60 1:30	8.19±0.85	2.15±0.47	14.86±1.64	UAE15 EA60 1:30	11.85±0.98	3.16±0.78	20.03±1.16	MAE8 EA60 1:30	13.95±0.94	3.71±0.53	23.90±1.68
CSLE60 EA80 1:30	9.17±0.83	2.63±0.66	18.13±1.88	UAE15 EA80 1:30	13.31±1.03	3.55±0.83	22.51±1.23	MAE8 EA80 1:30	15.67±1.08	4.17±0.71	26.86±1.32
CSLE60 EA90 1:30	7.72±0.88	2.48±0.72	15.60±1.63	UAE15 EA90 1:30	9.72±0.79	2.59±0.61	16.43±0.88	MAE8 EA90 1:30	11.16±0.91	2.97±0.63	19.12±1.33
CSLE60 EA100 1:30	6.51±0.61	1.92±0.48	12.79±1.34	UAE15 EA100 1:30	7.48±0.57	1.99±0.55	12.65±0.54	MAE8 EA100 1:30	8.70±0.54	2.31±0.51	14.92±1.38
CSLE90 EA60 1:30	4.48±0.48	1.56±0.39	8.37±0.86	UAE20 EA60 1:30	6.39±0.49	2.16±0.49	11.87±0.67	MAE10 EA60 1:30	7.94±0.43	2.58±0.55	14.27±1.28
CSLE90 EA80 1:30	5.01±0.52	1.91±0.42	10.22±1.06	UAE20 EA80 1:30	7.18±0.66	2.42±0.53	13.34±0.63	MAE10 EA80 1:30	8.92±0.61	2.89±0.47	16.03±1.16
CSLE90 EA90 1:30	4.22±0.44	1.80±0.38	8.79±0.89	UAE20 EA90 1:30	5.24±0.58	1.77±0.43	9.74±0.48	MAE10 EA90 1:30	6.35±0.55	2.06±0.46	11.42±1.24
CSLE90 EA100 1:30	3.49±0.42	1.40±0.37	7.21±0.76	UAE20 EA100 1:30	4.03±0.49	1.36±0.37	7.49±0.42	MAE10 EA100 1:30	4.96±0.39	1.61±0.56	8.91±0.86

Table 4SM. Experimental conditions for conventional solid-liquid extraction (CSLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) presented as variable codes used in Principal Components Analysis

Type of extraction	No.	Sample codes	Variable codes			Experimental values		
			Time (min)	Concentration (%) **(A)**	Ratio (w:v) **(B)**	Time (min)	Concentration (%) **(A)**	Ratio (w:v) **(B)**
CSLE	1	$C_{-2}A_2B_0$	– 2	2	0	15	100	1:15
	2	$C_{-1}A_2B_0$	– 1	2	0	30	100	1:15
	3	$C_0A_2B_0$	0	2	0	45	100	1:15
	4	$C_1A_2B_0$	1	2	0	60	100	1:15
	5	$C_2A_2B_0$	2	2	0	90	100	1:15
	6	$C_{-2}A_1B_0$	– 2	1	0	15	90	1:15
	7	$C_{-1}A_1B_0$	– 1	1	0	30	90	1:15
	8	$C_0A_1B_0$	0	1	0	45	90	1:15
	9	$C_1A_1B_0$	1	1	0	60	90	1:15
	10	$C_2A_1B_0$	2	1	0	90	90	1:15
	11	$C_{-2}A_0B_0$	– 2	0	0	15	80	1:15
	12	$C_{-1}A_0B_0$	– 1	0	0	30	80	1:15
	13	$C_0A_0B_0$	0	0	0	45	80	1:15
	14	$C_1A_0B_0$	1	0	0	60	80	1:15
	15	$C_2A_0B_0$	2	0	0	90	80	1:15
	16	$C_{-2}A_{-1}B_0$	– 2	– 1	0	15	70	1:15
	17	$C_{-1}A_{-1}B_0$	– 1	– 1	0	30	70	1:15
	18	$C_0A_{-1}B_0$	0	– 1	0	45	70	1:15
	19	$C_1A_{-1}B_0$	1	– 1	0	60	70	1:15
	20	$C_2A_{-1}B_0$	2	– 1	0	90	70	1:15
	21	$C_{-2}A_{-2}B_0$	– 2	– 2	0	15	60	1:15
	22	$C_{-1}A_{-2}B_0$	– 1	– 2	0	30	60	1:15
	23	$C_0A_{-2}B_0$	0	– 2	0	45	60	1:15
	24	$C_1A_{-2}B_0$	1	– 2	0	60	60	1:15
	25	$C_2A_{-2}B_0$	2	– 2	0	90	60	1:15
	26	$C_{-2}A_2B_1$	– 2	2	1	15	100	1:30
	27	$C_{-1}A_2B_1$	– 1	2	1	30	100	1:30
	28	$C_0A_2B_1$	0	2	1	45	100	1:30
	29	$C_1A_2B_1$	1	2	1	60	100	1:30
	30	$C_2A_2B_1$	2	2	1	90	90	1:30
	31	$C_{-2}A_1B_1$	– 2	1	1	15	90	1:30
	32	$C_{-1}A_1B_1$	– 1	1	1	30	90	1:30
	33	$C_0A_1B_1$	0	1	1	45	90	1:30
	34	$C_1A_1B_1$	1	1	1	60	90	1:30
	35	$C_2A_1B_1$	2	1	1	90	80	1:30
	36	$C_{-2}A_0B_1$	– 2	0	1	15	80	1:30
	37	$C_{-1}A_0B_1$	– 1	0	1	30	80	1:30
	38	$C_0A_0B_1$	0	0	1	45	80	1:30
	39	$C_1A_0B_1$	1	0	1	60	80	1:30
	40	$C_2A_0B_1$	2	0	1	90	70	1:30
	41	$C_{-2}A_{-1}B_1$	– 2	– 1	1	15	70	1:30
	42	$C_{-1}A_{-1}B_1$	– 1	– 1	1	30	70	1:30
	43	$C_0A_{-1}B_1$	0	– 1	1	45	70	1:30
	44	$C_1A_{-1}B_1$	1	– 1	1	60	70	1:30
	45	$C_2A_{-1}B_1$	2	– 1	1	90	60	1:30

	46	$C_{-2}A_{-2}B_1$	− 2	− 2	1	15	60	1:30
	47	$C_{-1}A_{-2}B_1$	− 1	− 2	1	30	60	1:30
	48	$C_0A_{-2}B_1$	0	− 2	1	45	60	1:30
	49	$C_1A_{-2}B_1$	1	− 2	1	60	60	1:30
	50	$C_2A_{-2}B_1$	2	− 2	1	90	60	1:30
UAE	51	$U_{-2}A_2B_0$	− 2	2	0	3	100	1:15
	52	$U_{-1}A_2B_0$	− 1	2	0	5	100	1:15
	53	$U_0A_2B_0$	0	2	0	10	100	1:15
	54	$U_1A_2B_0$	1	2	0	15	100	1:15
	55	$U_2A_2B_0$	2	2	0	20	100	1:15
	56	$U_{-2}A_1B_0$	− 2	1	0	3	90	1:15
	57	$U_{-1}A_1B_0$	− 1	1	0	5	90	1:15
	58	$U_0A_1B_0$	0	1	0	10	90	1:15
	59	$U_1A_1B_0$	1	1	0	15	90	1:15
	60	$U_2A_1B_0$	2	1	0	20	90	1:15
	61	$U_{-2}A_0B_0$	− 2	0	0	3	80	1:15
	62	$U_{-1}A_0B_0$	− 1	0	0	5	80	1:15
	63	$U_0A_0B_0$	0	0	0	10	80	1:15
	64	$U_1A_0B_0$	1	0	0	15	80	1:15
	65	$U_2A_0B_0$	2	0	0	20	80	1:15
	66	$U_{-2}A_{-1}B_0$	− 2	− 1	0	3	70	1:15
	67	$U_{-1}A_{-1}B_0$	− 1	− 1	0	5	70	1:15
	68	$U_0A_{-1}B_0$	0	− 1	0	10	70	1:15
	69	$U_1A_{-1}B_0$	1	− 1	0	15	70	1:15
	70	$U_2A_{-1}B_0$	2	− 1	0	20	70	1:15
	71	$U_{-2}A_{-2}B_0$	− 2	− 2	0	3	60	1:15
	72	$U_{-1}A_{-2}B_0$	− 1	− 2	0	5	60	1:15
	73	$U_0A_{-2}B_0$	0	− 2	0	10	60	1:15
	74	$U_1A_{-2}B_0$	1	− 2	0	15	60	1:15
	75	$U_2A_{-2}B_0$	2	− 2	0	20	60	1:15
	76	$U_{-2}A_2B_1$	− 2	2	1	3	100	1:30
	77	$U_{-1}A_2B_1$	− 1	2	1	5	100	1:30
	78	$U_0A_2B_1$	0	2	1	10	100	1:30
	79	$U_1A_2B_1$	1	2	1	15	100	1:30
	80	$U_2A_2B_1$	2	2	1	20	90	1:30
	81	$U_{-2}A_1B_1$	− 2	1	1	3	90	1:30
	82	$U_{-1}A_1B_1$	− 1	1	1	5	90	1:30
	83	$U_0A_1B_1$	0	1	1	10	90	1:30
	84	$U_1A_1B_1$	1	1	1	15	90	1:30
	85	$U_2A_1B_1$	2	1	1	20	80	1:30
	86	$U_{-2}A_0B_1$	− 2	0	1	3	80	1:30
	87	$U_{-1}A_0B_1$	− 1	0	1	5	80	1:30
	88	$U_0A_0B_1$	0	0	1	10	80	1:30
	89	$U_1A_0B_1$	1	0	1	15	80	1:30
	90	$U_2A_0B_1$	2	0	1	20	70	1:30
	91	$U_{-2}A_{-1}B_1$	− 2	− 1	1	3	70	1:30
	92	$U_{-1}A_{-1}B_1$	− 1	− 1	1	5	70	1:30
	93	$U_0A_{-1}B_1$	0	− 1	1	10	70	1:30
	94	$U_1A_{-1}B_1$	1	− 1	1	15	70	1:30
	95	$U_2A_{-1}B_1$	2	− 1	1	20	60	1:30
	96	$U_{-2}A_{-2}B_1$	− 2	− 2	1	3	60	1:30
	97	$U_{-1}A_{-2}B_1$	− 1	− 2	1	5	60	1:30
	98	$U_0A_{-2}B_1$	0	− 2	1	10	60	1:30
	99	$U_1A_{-2}B_1$	1	− 2	1	15	60	1:30
	100	$U_2A_{-2}B_1$	2	− 2	1	20	60	1:30

MAE	101	$M_{-2}A_2B_0$	− 2	2	0	1	100	1:15
	102	$M_{-1}A_2B_0$	− 1	2	0	3	100	1:15
	103	$M_0A_2B_0$	0	2	0	5	100	1:15
	104	$M_1A_2B_0$	1	2	0	8	100	1:15
	105	$M_2A_2B_0$	2	2	0	10	100	1:15
	106	$M_{-2}A_1B_0$	− 2	1	0	1	90	1:15
	107	$M_{-1}A_1B_0$	− 1	1	0	3	90	1:15
	108	$M_0A_1B_0$	0	1	0	5	90	1:15
	109	$M_1A_1B_0$	1	1	0	8	90	1:15
	110	$M_2A_1B_0$	2	1	0	10	90	1:15
	111	$M_{-2}A_0B_0$	− 2	0	0	1	80	1:15
	112	$M_{-1}A_0B_0$	− 1	0	0	3	80	1:15
	113	$M_0A_0B_0$	0	0	0	5	80	1:15
	114	$M_1A_0B_0$	1	0	0	8	80	1:15
	115	$M_2A_0B_0$	2	0	0	10	80	1:15
	116	$M_{-2}A_{-1}B_0$	− 2	− 1	0	1	70	1:15
	117	$M_{-1}A_{-1}B_0$	− 1	− 1	0	3	70	1:15
	118	$M_0A_{-1}B_0$	0	− 1	0	5	70	1:15
	119	$M_1A_{-1}B_0$	1	− 1	0	8	70	1:15
	120	$M_2A_{-1}B_0$	2	− 1	0	10	70	1:15
	121	$M_{-2}A_{-2}B_0$	− 2	− 2	0	1	60	1:15
	122	$M_{-1}A_{-2}B_0$	− 1	− 2	0	3	60	1:15
	123	$M_0A_{-2}B_0$	0	− 2	0	5	60	1:15
	124	$M_1A_{-2}B_0$	1	− 2	0	8	60	1:15
	125	$M_2A_{-2}B_0$	2	− 2	0	10	60	1:15
	126	$M_{-2}A_2B_1$	− 2	2	1	1	100	1:30
	127	$M_{-1}A_2B_1$	− 1	2	1	3	100	1:30
	128	$M_0A_2B_1$	0	2	1	5	100	1:30
	129	$M_1A_2B_1$	1	2	1	8	100	1:30
	130	$M_2A_2B_1$	2	2	1	10	90	1:30
	131	$M_{-2}A_1B_1$	− 2	1	1	1	90	1:30
	132	$M_{-1}A_1B_1$	− 1	1	1	3	90	1:30
	133	$M_0A_1B_1$	0	1	1	5	90	1:30
	134	$M_1A_1B_1$	1	1	1	8	90	1:30
	135	$M_2A_1B_1$	2	1	1	10	80	1:30
	136	$M_{-2}A_0B_1$	− 2	0	1	1	80	1:30
	137	$M_{-1}A_0B_1$	− 1	0	1	3	80	1:30
	138	$M_0A_0B_1$	0	0	1	5	80	1:30
	139	$M_1A_0B_1$	1	0	1	8	80	1:30
	140	$M_2A_0B_1$	2	0	1	10	70	1:30
	141	$M_{-2}A_{-1}B_1$	− 2	− 1	1	1	70	1:30
	142	$M_{-1}A_{-1}B_1$	− 1	− 1	1	3	70	1:30
	143	$M_0A_{-1}B_1$	0	− 1	1	5	70	1:30
	144	$M_1A_{-1}B_1$	1	− 1	1	8	70	1:30
	145	$M_2A_{-1}B_1$	2	− 1	1	10	60	1:30
	146	$M_{-2}A_{-2}B_1$	− 2	− 2	1	1	60	1:30
	147	$M_{-1}A_{-2}B_1$	− 1	− 2	1	3	60	1:30
	148	$M_0A_{-2}B_1$	0	− 2	1	5	60	1:30
	149	$M_1A_{-2}B_1$	1	− 2	1	8	60	1:30
	150	$M_2A_{-2}B_1$	2	− 2	1	10	60	1:30

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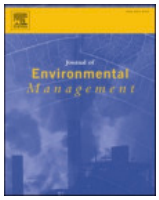
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Research article



Subcritical water extraction of bioactive phenolic compounds from distillery stillage

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ABSTRACT

Distilleries generate huge amounts of by-products that have a negative impact on the environment, so the management of wastes generated by this sector should be improved. Because distillery by-products are a source of bioactive compounds, the recovery of these compounds not only reduces issues with environmental protection but also provides the basis for a waste-to-profit solution. Following the latest trends in the search for so-called green extraction techniques for recovering valuable products, this study investigated the effect of subcritical water extraction (SWE) conditions (temperature (25–260 °C), time (5–90 min), and solid-to-solvent ratio (1:5–1:50, w:v)) on the efficiency of recovery of bioactive compounds, i.e., polyphenols from distillery stillage, and on the antioxidant activity of the extracts. The highest extraction yield was obtained with 30-min SWE with a solid-to-solvent ratio of 1:15 at either 140 °C (for total polyphenol content and phenolic acid content) or 200 °C (for total flavonoid content), as indicated by the Response Surface Methodology analysis. Phenolic acids in the extracts were present mainly in free forms (up to 88% of the total content). The antioxidant activity, which was measured using several assays, correlated positively with the content of phenolic acids, which confirmed their significant contribution to the bioactive properties of the extracts. The antioxidant effects of the extracts were mostly due to hydroxycinnamic acids (especially ferulic and p-coumaric acids). Principal component analysis showed that the temperature and time of SWE were the factors that can explain the greatest amount of variation in the extraction yield, composition, and bioactive properties of the polyphenols. These results will influence the design of further processes, such as purification and concentration, which are necessary before using the extracted compounds as substrates that are applicable in various industries. Based on the analysis of the elemental composition, the biomass remaining after SWE was evaluated to consider the possibilities of its further utilization.

1. Introduction

The distillery industry contributes significantly to the global economy, while at the same time generating huge amounts of by-products (termed distillery stillage). For 1 L of alcohol produced, about 15 L of distillery stillage is generated, which is characterized by a low pH, a high content of organic matter, and a dark brown color (Ratna et al., 2021). In 2020, about 117 billion liters of alcohol were produced worldwide (Sonnichsen, 2021), but the rate at which it is being consumed is constantly growing, particularly concerning medical and fuel purposes. Therefore, it is important to develop new strategies to manage distillery stillage, for example, to promote a circular economy. The concept of a circular economy includes the valorization of by-products *via* the recovery of compounds with valuable functions. Industrial cereal by-products, which are generated during alcohol production, are rich in bioactive compounds (polyphenols) with potential health benefits (Dzah et al., 2020). Thus, polyphenol recovery from distillery stillage may prove to be an innovative approach to not only reducing the pollution generated during alcohol production but also increasing the economic competitiveness of the distillery industry.

The most common phenolic compounds (polyphenols) found in cereal by-products are phenolic acids and flavonoids (Liu, 2007). The basic structure of flavonoids comprises a flavonoid nucleus containing 15 carbon atoms arranged in three rings (C6–C3–C6). Flavonoids exist mainly as glycosides and polymers. Phenolic acids are hydroxy derivatives of aromatic carboxylic acids, which are derivatives of benzoic or cinnamic acids (Kim et al., 2006) and are connected to the structural components of the cell wall, such as cellulose, lignin, tannins and

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proteins. In acidic conditions, these bound structures may undergo hydrolysis (Liu, 2007). In addition, industrial processes, such as heat treatment, pasteurization, fermentation, and freezing, contribute to the release of phenolic compounds (Dewanto et al., 2002). Therefore, an approach that uses by-products from alcohol production as a rich and cheap source of phenolic compounds may be of particular interest.

To meet the requirements of sustainable development, the recovery of polyphenolic compounds should be carried out using green extraction techniques that minimize the volumes of solvents and avoid the use of toxic substances (Chemat et al., 2012). Therefore, in recent years, ultrasound- and microwave-assisted extractions, high-pressure processing, or subcritical water extraction (SWE) have been developed (Barba et al., 2016). Among these techniques, SWE has turned out to be an environmentally-friendly alternative because it uses solvents at temperatures above their atmospheric boiling point and the elevated pressure maintains the solvent in its liquid state (Attard and Hunt, 2018). At room temperature and atmospheric pressure, water is an insufficiently effective solvent as a result of its high polarity and high dielectric constant. Under SWE conditions, with pressure sufficient to maintain water in the liquid phase at increased temperature, the dielectric constant and polarity of water decrease (Teo et al., 2010). This causes water to have the properties of organic solvents under SWE conditions, which is highly suitable for the extraction of phenolic compounds. Because the dielectric constant and polarity of solvents are critical parameters that determine SWE efficiency, it is important to optimize SWE conditions in terms of the yield of recovered compounds. Moreover, water under SWE conditions exhibits hydrolytic properties, which enables the release of bound compounds and the breakdown of larger molecules while maintaining high biological and antioxidant activity (Plaza and Turner, 2015). In recent years, research has focused on the SWE of phenolic compounds with antioxidant activity from various by-products, such as rice bran (Pourali et al., 2010), grape pomace (Pedras et al., 2020), olive fruit dregs (Yu et al., 2015), and soybean residues (Nkurunziza et al., 2019). However, SWE has not yet been applied in the recovery of phenolic compounds from distillery stillage. When trying to optimize SWE as an effective and environmentally friendly procedure for the extraction of bioactive compounds from distillery by-products, it is important to obtain extracts with the highest possible antioxidant activity to support their potential use as an alternative to synthetic antioxidants in industry.

Phenolic compounds are natural antioxidants with antibacterial, antiviral, anticancer, and anti-inflammatory properties (Kumar and Goel, 2019). Recently, particular attention has been paid to research on the potential use of phenolic acids in the food, pharmaceutical, and cosmetics industries (Kalogerakis et al., 2013). These compounds have proven to be more effective than vitamin E and carotenoids for this purpose (Shahidi and Ambigaipalan, 2015). For this reason, they can replace synthetic antioxidants used in food products, such as butylated hydroxyanisole, butylated hydroxytoluene, tert-butylhydroquinone, or propyl gallate, which may be toxic and carcinogenic (Shahidi and Ambigaipalan, 2015).

The antioxidant activity of phenolic compounds depends on their ability to scavenge free radicals, interfere with radical chain reactions, and chelate metals. Due to the variety of these reactions, the antioxidant activity can be monitored by several simple and cheap methods which include the use of ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (iron-reducing antioxidants). These methods are based on hydrogen atom transfer (HAT) or single electron transfer (SET) (Awika et al., 2003). HAT is a single-step reaction that depends on the enthalpy of hydroxyl bond dissociation, while SET is a two-step reaction that depends on the ionization potential and proton dissociation enthalpy and is also influenced by the affinity for protons and the electron transfer enthalpy (Mazzone et al., 2015). These reaction mechanisms may occur independently or simultaneously at different rates depending on the raw material and the environmental conditions under which the bioactive compounds are present (Shadnia and Wright, 2008). Moreover, the structure of antioxidants is strongly related to their activity. In the case of phenolic acids, the antioxidant activity is directly influenced by phenolic hydroxyl, methoxy, and carboxyl groups. Depending on the raw material and the conditions of polyphenolic compound recovery, their activity also depends on intermolecular interactions, which may be antagonistic or synergistic (Ammar et al., 2009). Therefore, to demonstrate the relationship between the structure of the polyphenols from a newly investigated material (e.g., distillery stillage) and their antioxidant activity, it is advisable to carry out several types of tests to determine the antioxidant activity of the recovered bioactive compounds.

Based on a literature review, it seems that there is a lack of information on the recovery of high-quality bioactive compounds from distillery stillage employing green extraction procedures. Therefore, this study focused on the recovery of polyphenols using SWE, intending to achieve the highest yield of the targeted compounds and maintain their antioxidant activity. The specific objectives of the study were to determine the effects of the temperature, extraction time, and solid-to-solvent ratio on (i) total polyphenol content (TPC) and total flavonoid content (TFC) in the extracts, (ii) the types and content of free and bound phenolic acids, (iii) the extraction kinetics, and (iv) the antioxidant activity of extracts. To simultaneously evaluate the effects of the experimental variables on polyphenol extraction, Response Surface Methodology (RSM) was used. To examine relationships between the extraction parameters and the antioxidant activity of the recovered compounds, principal component analysis (PCA) was performed. The elemental composition of the biomass remaining after SWE was evaluated. This study provides information that will enable the industry to efficiently manage distillery by-products by recovering value-added compounds while maintaining the high functional quality of these compounds.

2. Materials and methods

2.1. Distillery stillage samples

This study used distillery stillage collected from a company in northeastern Poland and generated during the production of concentrated raw ethyl alcohol from cereals (mainly wheat and rye). The stillage samples had the following characteristics: 47,000 ± 5300 mg COD/L, 4345 ± 5 mg N_{tot}/L, 280 ± 2 mg P_{tot}/L, 789 ± 3 mg CH_3COOH/L, a pH of 4.52, 63.8 g dry matter (DM)/kg, and 59.5 g organic dry matter (ODM)/kg. Immediately after transport to the laboratory, the samples were freeze-dried (STERIS Lyovac GT2 freeze dryer with a Leybold Trivac vacuum pump, SRK-Systemtechnik GmbH, Germany). The freeze-dried sample had the following characteristics: 901.8 g DM/kg and 864.7 g ODM/kg. To produce 1 g of freeze-dried sample, 15.3 g of distillery stillage was used. The TPC, TFC, contents of phenolic acids and antioxidant activities were expressed per gram DM of distillery stillage before freeze-drying.

2.2. Extraction conditions

SWE was performed using a microwave system (MARSXpress 240/50, CEM Corporation, USA) equipped with a temperature (RTP-300 Plus sensor, CEM Corporation, USA) and pressure (ESP-1500 Plus sensor, CEM Corporation, USA) control systems. Extractions were carried out for 5, 15, 30, 60, and 90 min with temperatures (T) of 25, 80, 140, 200, and 260 °C, a pressure of 4.14 MPa, and a microwave frequency of 2.45 GHz. Distilled water was used as the solvent. In each extraction, solid-to-solvent ratios (r) of 1:5, 1:15, 1:30, and 1:50 (w:v) were tested. The samples were placed into Teflon Easy Prep vessels (CEM Corporation, USA) with a volume of 115 mL which were equipped with a sealing mechanism. After extraction, samples were centrifuged (MPW-380 Centrifuge, Donserv, Poland) for 10 min at 10,000 rpm. In the extracts, TPC, TFC, concentrations of phenolic acids, antioxidant activity, and surface tension (ST) (K100C tensiometer, Kruss GmbH, Germany) were measured.

2.3. Quantification of TPC and TFC

TPC was measured with Folin-Ciocalteu reagent (Sigma-Aldrich, USA) based on Singletion et al. (1999). TFC was measured using aluminum chloride based on Quettier-Deleu et al. (2000). The absorbances were measured with a UV–Vis spectrophotometer (Cary 50 Scan UV–Visible, Varian, Australia). TPC was expressed as milligrams of gallic acid equivalent (mg GAE/g DM); and TFC, as milligrams of quercetin equivalent (mg QUE/g DM). Gallic acid and quercetin for curve calibration were obtained from Sigma-Aldrich (USA).

2.4. Total and free phenolic acids

To determine the content of total phenolic acids in distillery stillage, acid hydrolysis was conducted. A 1 g distillery stillage sample was mixed with 2 M NaOH for 4 h. Then, the solution was acidified with 6 M HCl (to pH 2), and the sample was centrifuged (Centrifuge MPW-380, Donserv, Poland) for 10 min at 10,000 rpm. The total phenolic acids were extracted 4 times with diethyl ether.

To determine the content of free phenolic acids, the extracts were evaporated to dryness in a vacuum evaporator (R-210, BUCHI Labortechnik, Switzerland) at 50 °C. The dry solids were dissolved in 30 mL of deionized water acidified to pH 2 with 6 M HCl. The free phenolic acids were extracted (liquid-to-liquid) from the aqueous solution with 20 mL of ethyl acetate. Finally, the diethyl ether extracts with total and free phenolic acids were concentrated on a rotary vacuum evaporator (R-210, BUCHI Labortechnik, Switzerland) at 50 °C and re-dissolved in 1 mL of methanol (purity: 99.9%, Sigma-Aldrich, USA).

2.5. Identification of phenolic acids by HPLC

The samples prepared as given in section 2.4 were analyzed *via* HPLC (ProStar 325, Varian, Australia) equipped with an autosampler (ProStar 410, Varian, Australia) and coupled with a UV–Vis detector (ProStar 325, Varian, Australia), according to Mikucka et al. (2022).

2.6. Evaluation of antioxidant activity

Three spectrophotometric assays for determining antioxidant activity *in vitro* were performed: ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidants power). These assays are based on radical scavenging activity (ABTS and DPPH) or reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (FRAP).

The ABTS assay is based on $ABTS^{\bullet+}$ (radical cation) decolorization, which was conducted according to Re et al. (1999). The $DPPH^{\bullet}$ radical scavenging of samples was determined according to the procedure described by Moure et al. (2001). The results of both assays were expressed as μmol of Trolox equivalent (μmol TE/g DM). The FRAP assay was performed according to the chemical assay for reducing iron developed by Benzie and Strain (1996). The results were expressed as μmol $FeSO_4$ equivalent (μmol $FeSO_4$/g DM). All reagents came from Sigma-Aldrich (USA). The absorbances were measured with the UV–Vis spectrophotometer (Cary 50 Scan UV–Visible, Varian, Australia).

2.7. Data processing and statistical analysis

Response Surface Methodology (RSM) used a three-factor composite design to optimize SWE conditions to achieve the highest polyphenol content, using Statistica 13.1 (StatSoft). The variables that were used were: temperature (25, 80, 140, 200, and 260 °C; X_1), extraction time (5, 15, 30, 60, and 90 min; X_2), and solid-to-solvent ratio (1:5, 1:15, 1:30 and 1:50 (w:v); X_3). The pressure of extraction and particle sizes were kept at constant values. The RSM was described with the following equation (Equation (1)):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_{ij} \quad (1)$$

where: Y – polyphenol content, X_i and X_j – independent variables; β_0 – constant-coefficient; β_i – linear-coefficient; β_{ii} – quadratic-coefficient; β_{ij} – cross-product coefficients (where: i = 1,2,3, …, k and j = 1, 2, 3, …, k); and k – number of variables.

Results were expressed as the means ± standard deviation of three measurements. Spearman's rank correlation (r_s) was employed to quantify correlations between TFC, TPC, concentrations of phenolic acids, ABTS, FRAP, and DPPH and extraction conditions. Principal Component Analysis (PCA) and a correlation matrix were calculated with R Version February 1, 1335. The correlation matrix was visualized with a correlogram using the "corrplot" package. PCA was plotted with the "ggplot2" and "ggfortify" packages. To create a heatmap of the individual phenolic acid contents in distillery stillage extracts obtained with SWE, the "heatmap.2" and "gplots" packages for R were used. Other data processing and statistical analyses were performed with Statistica 13.1 (StatSoft), and all results were regarded as statistically significant at $p < 0.05$.

A second-order kinetic model was used for determining the rate of extraction of phenolic acids from distillery stillage, based on Xie et al. (2019). The fit of the model to the experimental data was evaluated using the coefficient of determination (R^2). Equations (2) and (3) were used.

$$C_t = \frac{k \cdot t \cdot C_e^2}{1 + k \cdot t \cdot C_e} \quad (2)$$

$$ER = k \cdot C_e^2 \quad (3)$$

where: C_t – concentration of phenolic acids (μg/g DM) in the extract at extraction time t (min), k – rate constant for the extraction (g DM/(μg·min)); C_e – equilibrium concentration of the compound (μg/g DM) in the extract; ER – extraction rate (μg/(g DM·min)).

2.8. Investigations of the biomass remaining after SWE

In the biomass remaining after SWE, the contents of DM, ash and ODM were measured (APHA, 1992). After freeze-drying (with the same procedure as in section 2.1), the elemental composition of this biomass was analyzed with a FLASH 2000 Series Thermo (USA) elemental analyzer at 960 °C, employing a thermal conductivity detector and helium as a carrier gas. The analyzer reported the contents of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S), and the oxygen content (O) was calculated according to Gamgoum et al. (2016):

$$O\% = 100\% - C\% - H\% - N\% - S\% - ash\% \quad (4)$$

Based on the elemental analysis, the higher heating value (HHV) and energy densification were calculated according to Deng et al. (2022):

$$HHV\ (MJ/kg\ VS) = 337C + 1419(H - 0.125O) + 93S + 23.26N \quad (5)$$

$$\text{Energy densification ratio} = \text{HHV of solid residue/HHV of distillery stillage} \quad (6)$$

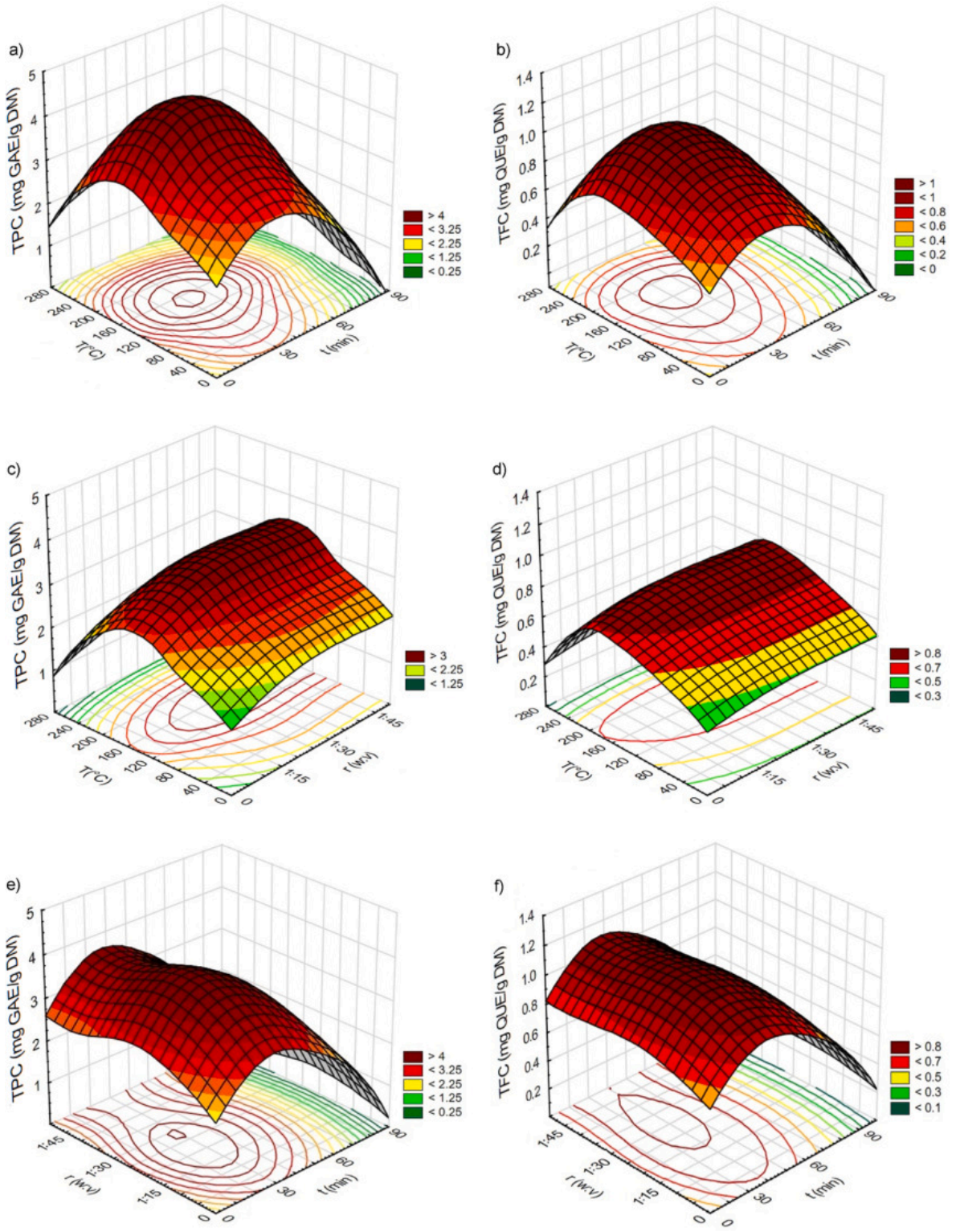


Fig. 1. Representative 3D plots showing the effects of pairs of variables (temperature (T), time (t), or solid-to-solvent ratio (r)) on the response variables TPC and TFC.

3. Results and discussion

3.1. TPC and TFC obtained with SWE

To determine the effects of the solid-to-solvent ratio, extraction time, and temperature on TPC and TFC in the SWE extracts, RSM was performed using polynomial equations (Fig. 1). In all cases, wavy surfaces were obtained, which indicated that all the variables had a substantial influence on the extractions within the explored range.

3.1.1. Effect of solid-to-solvent ratio on TPC and TFC

The extraction yield of polyphenols from distillery stillage was highest at the *r* of 1:15 (w:v). TPC and TFC in extracts increased 1.19-times and 1.21-times, respectively, when the *r* was changed from 1:5

to 1:15 (w:v) (Fig. 1c–f). These results are consistent with what would be expected based on mass transfer rules, which state that the driving force for mass transfer is the gradient between solids and solvents (Tan et al., 2011). A higher *r* increases the concentration gradient, leading to an increase in the diffusion rate of compounds from the extracted solid to the solvent, leading to a sharp, exponential increase in extraction yield followed by equilibrium (Durdun et al., 2016). In addition, the heating, which facilitates the extraction, is faster when a larger volume of solvent is used in a closed-vessel system of microwave extraction (Letellier and Budzinski, 1999). Although the extraction of phenolic compounds generally increases with an increasing proportion of solvent to solids, the increase in the yield of phenolic compounds may not be directly proportional (Tan et al., 2011). This was noted in the present study when the polyphenol extraction yield increased negligibly after changing *r* from 1:15 to 1:30 (w:v). At *r* values of 1:15 and 1:30 (w:v), similar TPC (4.88 ± 0.16 and 4.93 ± 0.21 mg GAE/g DM, respectively) and TFC (1.24 ± 0.23 and 1.25 ± 0.16 mg QUE/g DM, respectively) yields were obtained. These results can be explained by the fact that, after reaching an equilibrium state, an increase in the amount of solvent does not increase the extraction efficiency. However, a further change in the *r* value to 1:50 (w:v) resulted in a slight decrease in TPC and TFC. The use of a high proportion of solvents to solids causes the solution to be diluted (Ho et al., 2008), which explains the decrease in TPC and TFC obtained in the present study. To sum up, in the present study, an *r* value of 1:15 (w:v) was found to be the most beneficial for the extraction of phenolic compounds from distillery stillage. It is important, therefore, to evaluate the effect of the *r* when optimizing an extraction, since it allows efficient use of the solvent.

3.1.2. Effect of extraction times on TPC and TFC

The extraction time is an important factor in the extraction of phenolic compounds because properly selected it can maximize the efficiency of extraction. Extending the SWE time from 5 to 30 min increased the extracted TPC from 3.04 ± 0.11 to 4.88 ± 0.16 mg GAE/g DM (Fig. 1ae) and the TFC from 0.72 ± 0.08 to 1.24 ± 0.23 mg QUE/g DM (Fig. 1bf). Further extending the SWE duration to 90 min gradually decreased the extraction efficiency. The polyphenol content extracted by 90-min SWE was lower than that extracted by 5-min SWE, in terms of both TPC (1.46-times) and TFC (1.32-times). The TPC and TFC obtained with the tested extraction times decreased in the following order: 30 min > 60 min >15 min > 5 min >90 min. Thus, too long an extraction time was not suitable for the extraction of the phenolic compounds. It was observed that, in each temperature variant, 30-min SWE recovered the most polyphenols (Fig. 1 abef). During extraction, the cell wall matrix breaks, increasing the contact surface of the solvent with the solids, which leads to an increase in the mass transfer rate (Xu et al., 2016). This behavior may be related to an increase in hydrogen bond decay in longer extraction times, which increases the solubility of phenolic compounds (Kheirkhah et al., 2019). However, an excessively long process time, which in the presented study was above 30 min, may reduce the content of polyphenols in the extracts because they lose chemical stability and are oxidized and degraded. Long process times cause the excessive formation of free radicals, which may affect the chemical structure of the polyphenols, significantly reducing their antioxidant properties (Xie et al., 2019). In addition, attention should be paid to the fact that an excessively long extraction process may cause oxidation and polymerization of other compounds (Shahidi and Naczk, 1995), thus creating products that may significantly reduce the desired properties of the extract.

3.1.3. Effect of extraction temperature on TPC and TFC

In the present study, the pressure of SWE was high (4.14 MPa) and constant, and changing the water properties to increase the polyphenol yield was conducted by adjusting the extraction temperature. Increasing the SWE temperature from 25 to 140 °C significantly increased the TPC yield from 3.12 ± 0.09 to 4.88 ± 0.16 mg GAE/g DM (Fig. 1ac). However, a further increase in temperature from 140 to 200 °C decreased TPC extraction by about 11%. Continuing to increase the temperature to 260 °C significantly reduced TPC in the extracts by about 43% compared with SWE at 140 °C. Overall, 90-min SWE at 260 °C was the least efficient method of TPC extraction, with yields 1.82-times lower than 90-min SWE at 140 °C and 1.69-times lower than 90-min SWE at 25 °C.

The greatest increase in the TFC in the extracts (from 0.75 ± 0.12 to 1.24 ± 0.23 mg QUE/g DM) was obtained when SWE temperature was increased from 25 °C to 200 °C (Fig. 1bd). A further increase in temperature caused a significant decrease in TFC to 0.54 ± 0.26 mg QUE/g DM at 260 °C. As was the case with TPC, 90-min SWE at 260 °C was the least efficient option for extracting TFC (0.27 ± 0.14 mg QUE/g DM). Moreover, TFC extraction in this variant was lower than with 5-min SWE at 260 °C (0.43 ± 0.08 mg QUE/g DM) (Fig. 1bd).

These results show that the temperature had the greatest effect on the recovery of polyphenols, followed by the extraction time. TPC and TFC obtained at the same temperature but with different extraction times were similar (4.88 ± 0.16 and 4.65 ± 0.22 mg GAE/g DM at 140 °C with 30-min and 60-min SWE, respectively; 1.24 ± 0.23 and 1.18 ± 0.16 mg QUE/g DM at 200 °C with 30-min and 60-min SWE, respectively) (Fig. 1ab). Temperature changes during 30-min SWE significantly affected the extraction of compounds (4.88 ± 0.16 mg GAE/g DM and 1.02 ± 0.14 mg QUE/g DM at 140 °C, and 4.38 ± 0.11 mg GAE/g DM and 1.24 ± 0.23 mg QUE/g DM at 200 °C). Other studies have also noted that temperature is a key parameter for optimization of SWE of phenolic acids from black pea (Mukhopadhyay et al., 2006), phenolic compounds from parsley (Luthria, 2008), and antioxidants from winery wastes (Aliakbarian et al., 2012). Low temperatures may reduce SWE efficiency due to insufficient solubilization of polyphenols as a result of lower molecular motion (Dent et al., 2013). The importance of temperature may be particularly attributable to the thermal instability of phenolic compounds at high temperatures (Calinoiu and Vodnar, 2020). However, SWE conditions in the present study could have allowed the dielectric constant of the solvent to be low enough to ensure that the temperature of the solvent will remain low to avoid thermal degradation of extracted compounds (Routray and Orsat, 2012).

Water under subcritical conditions changes its properties, such as the dielectric constant, viscosity, and diffusivity. Therefore, water can be used as a solvent in the recovery of value-added compounds due to its solubilizing properties at high temperatures. Under subcritical conditions, the dielectric constant of water decreases ($\varepsilon = 80$ at 25 °C and $\varepsilon =$ 36 at 190 °C) and becomes more similar to that of organic solvents such as methanol ($\varepsilon = 33$ at 25 °C) and ethanol ($\varepsilon = 24$ at 25 °C) (Zhang et al., 2019b). However, literature reports indicate that pure ethanol and pure methanol are not the most effective conventional solvents for polyphenolic compounds and that the addition of water improved the extraction efficiency (Mikucka et al., 2022), which could have been caused by the increase in the ε. This may explain why, in the present study, reducing the ε of water by heating it to 140 °C provided the best conditions for the recovery of polyphenols from distillery stillage (Fig. 1abcd).

In addition, changing the temperature changes the conditions of a chemical reaction. Increasing the water temperature causes the chemical equilibrium to lower the temperature, absorbing additional heat. This creates more hydronium and hydroxyl ions, thus increasing the ionic product of water, which is a multiplication of the concentrations of hydronium and hydroxyl ions. This implies that the ionic strength is higher than at ambient temperature and the pH is lower. For example, as the temperature increased from 25 to 250 °C, the dissociation constant increased from $1{\cdot}10^{-14}$ to $4.9{\cdot}10^{-12}$ and pH dropped from 7.0 to 5.5 (Plaza and Turner, 2015). These conditions induce hydrolysis of the bonds between the bioactive compounds and the solid matrix, which may release the compounds. At the same time, with increasing water temperature, the viscosity and ST decrease, while the diffusivity increases, which affects the mass transfer properties (Giombelli et al.,

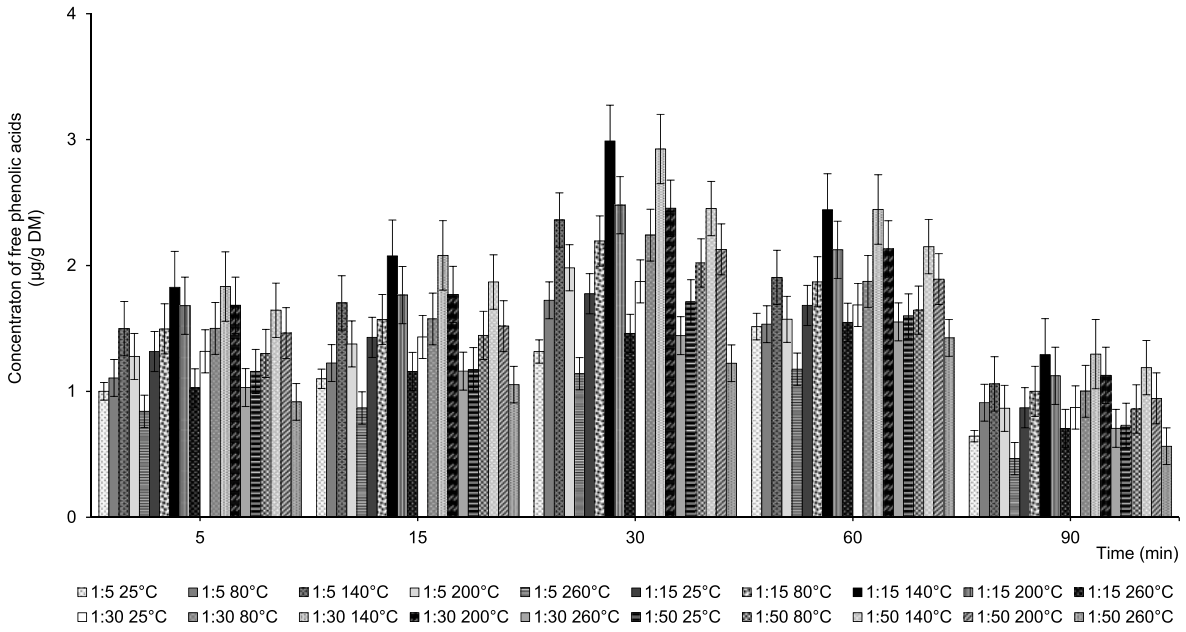


Fig. 2. Concentrations of free phenolic acids in extracts obtained with (a) 5-min, (b) 15-min, (c) 30-min, (d) 60-min, and (e) 90-min SWE.

2020). In the present study, the ST of the extracts ranged from 13.08 to 21.34 mN/m; the lowest ST values were obtained with 30-min SWE at 140 °C and 200 °C. This decrease in ST with increased extraction temperature favored penetration of the solvent into the sample matrix, which increased the contact surface between the matrix and the solvent (Pellati et al., 2013) and caused the faster mass transfer.

Despite the high thermal instability of polyphenols, SWE carried out in the present study showed a high yield of polyphenols at 140 °C. This was because at high temperatures the stillage matrix is more permeable, allowing faster transfer of polyphenols from the solid to the liquid phase, which can be explained by van der Waals's forces, hydrogen bonds, and dipole-dipole bonds (Giombelli et al., 2020). Insoluble phenolic

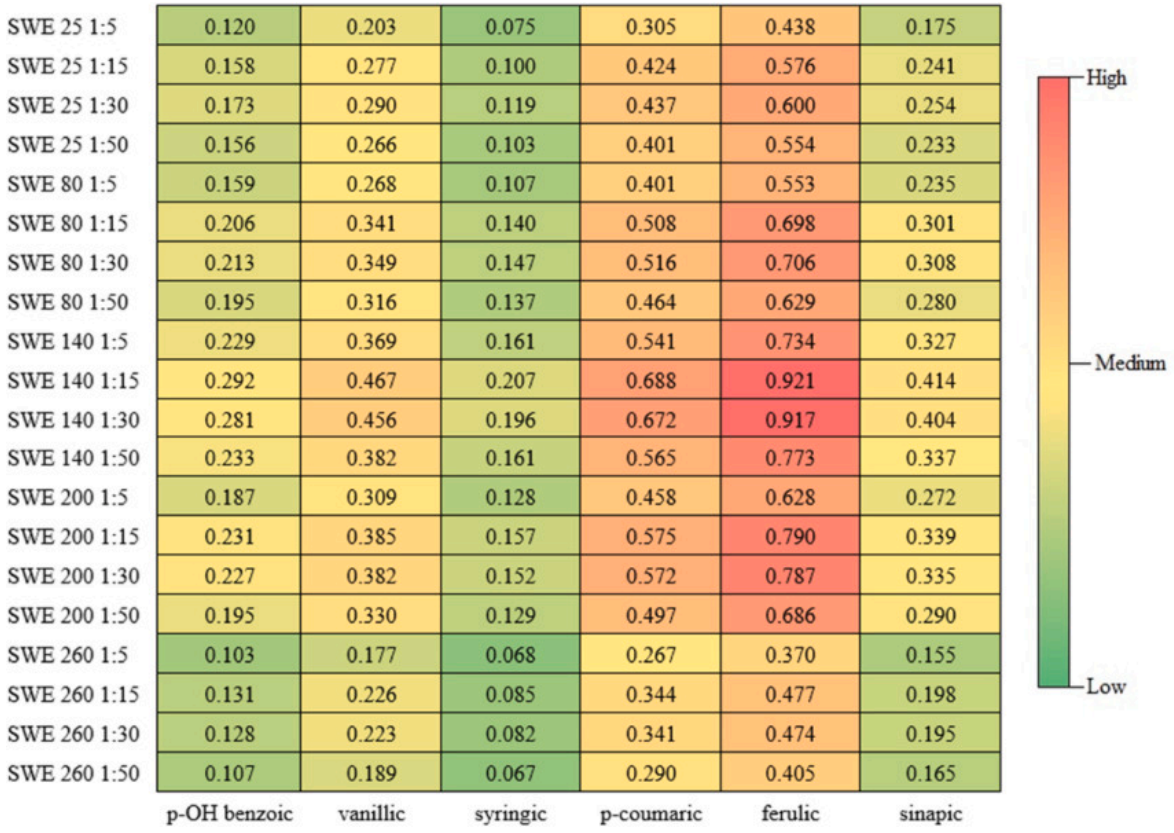


Fig. 3. Heatmap of the contents of individual phenolic acids (µg/g DM) in distillery stillage extracts obtained with 30-min SWE. In the abbreviations, the values after SWE indicate the extraction temperature followed by the *r* value. The content of individual phenolic acids is represented by a color (red indicates a high content; green indicates a low content). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Rate of SWE of individual and total free phenolic acids. In the abbreviations, the values after SWE indicate the temperature extraction; and finally, the solid-to-solvent ratio is given.

Type of extraction	Extraction rate (μg/(g DM·min))						
	p-OH benzoic	vanillic	syringic	p-coumaric	ferulic	sinapic	Total acids
SWE 25 1:5	0.045	0.066	0.030	0.105	0.140	0.049	0.450
SWE 80 1:5	0.049	0.079	0.034	0.113	0.149	0.057	0.481
SWE 140 1:5	0.079	0.101	0.048	0.162	0.219	0.077	0.685
SWE 200 1:5	0.056	0.082	0.041	0.132	0.174	0.060	0.546
SWE 260 1:5	0.046	0.069	0.037	0.107	0.152	0.056	0.471
SWE 25 1:15	0.065	0.097	0.045	0.152	0.209	0.076	0.634
SWE 80 1:15	0.077	0.112	0.052	0.174	0.238	0.087	0.753
SWE 140 1:15	0.086	0.146	0.067	0.305	0.658	0.106	1.333
SWE 200 1:15	0.082	0.123	0.058	0.187	0.256	0.091	0.780
SWE 260 1:15	0.046	0.074	0.036	0.116	0.154	0.058	0.472
SWE 25 1:30	0.060	0.091	0.039	0.145	0.207	0.071	0.594
SWE 80 1:30	0.082	0.122	0.055	0.193	0.256	0.090	0.786
SWE 140 1:30	0.084	0.119	0.058	0.218	0.264	0.098	0.953
SWE 200 1:30	0.082	0.121	0.055	0.181	0.244	0.088	0.815
SWE 260 1:30	0.046	0.074	0.036	0.116	0.154	0.058	0.472
SWE 25 1:50	0.048	0.078	0.035	0.118	0.158	0.058	0.491
SWE 80 1:50	0.063	0.091	0.045	0.141	0.196	0.067	0.624
SWE 140 1:50	0.082	0.108	0.052	0.184	0.242	0.095	0.832
SWE 200 1:50	0.070	0.102	0.048	0.160	0.218	0.079	0.652
SWE 260 1:50	0.047	0.070	0.034	0.111	0.152	0.057	0.470

compounds can be released due to the breaking of the lignin-phenolic acid bonds (Maillard and Berset, 1995). In addition, lignin itself can degrade at high temperatures. These may explain the increase in the efficiency of polyphenol extraction at high temperatures with SWE.

3.2. Identification and quantification of phenolic acids

3.2.1. Composition of phenolic acids

Extraction allows the recovery of phenolic acids that are in free form; therefore, to determine the total content of phenolic acids in the present study, hydrolysis was carried out, which allows the recovery of bound phenolic acids. It was observed that both after hydrolysis (extracts containing all phenolic acids) and after extraction without hydrolysis (extracts containing free phenolic acids), the profiles of the phenolic acids were similar. However, the content of free phenolic acids depended on the recovery conditions (Fig. 2).

The highest content of phenolic acids was present in the extracts obtained with 30-min SWE; the *r* values of 1:15 and 1:30 (w:v) gave similar results (Fig. 2). However, the temperature had the most important role in optimizing SWE for phenolic acid extraction, as was observed in the case of TPC. The concentration of phenolic acids was 1.8–2.3-times higher at 140 °C than at 260 °C. In the extracts, the content of free phenolic acids ranged from 0.47 to 2.99 μg/g DM (Fig. 2), while after hydrolysis, the average total content of phenolic acids was 3.39 ± 0.25 μg/g of DM. Free phenolic acids predominated in the extracts (up to 88% of the total in the most efficient extraction variant). The high proportion of free phenolic acids may have been caused by these acids that were released from the cereal matrix during the production of alcohol. Skrajda-Brdak et al. (2019) observed that, in the case of spelt and rye bread, sourdough fermentation increased the accumulation of free phenolic acids, while in wheat bread, this kind of fermentation decreased their accumulation. Thus, the processes taking place during fermentation affect the content of free phenolic acids; depending on the process conditions and the type of plant raw material, a decrease or increase in the level of free acids was observed (Tsopmo et al., 2014). Based on the observation in the present study that bound phenolic acids accounted for a small part of the total phenolic acid content in the extracts, it can be concluded that environmentally-friendly extraction with water under subcritical conditions can be successfully used for the recovery of phenolic acids from distillery stillage, without using chemicals for hydrolysis.

In the present study, 6 phenolic acids were identified: p-OH benzoic, p-coumaric, vanillic, syringic, sinapic, and ferulic. For their identification by HPLC, high-quality peak shapes, good separation, and minimal baseline variation were obtained (Fig. 1SM). Despite the determination of the optimal conditions for the extraction of polyphenols, it should be noted that the composition of phenolic acids and the content of individual phenolic acids in the extracts changed depending on their structure. Thus, to illustrate the differences in the contents of individual phenolic acids during 30-min SWE, a heat map was constructed (Fig. 3); the contents obtained with the other extraction time variants are shown in Fig. 2SM.

The concentration of the individual acids varied depending on the extraction conditions (Fig. 3). At lower temperatures (25 and 80 °C), a 6–10% higher content of individual acids was obtained with the *r* value of 1:30 (w:v) than one of 1:15 (w:v). However, at higher temperatures (140, 200, and 260 °C), higher contents were obtained with the *r* value of 1:15 (w:v). This change can be explained by the fact that, as the temperature increases, bound phenolic acids are released and the solubilizing properties of water increase, which results in faster mass transfer (as described in section 3.1.3).

In the extracts, ferulic and p-coumaric acids predominated, constituting from 29 to 32% and from 20 to 24% of all detected phenolic acids, respectively, depending on the extraction variant used (Fig. 3). In turn, p-OH benzoic, vanillic, and sinapic acids accounted for about 8–10%, 13–16%, and 12–14% of the total, respectively. Syringic acid had the smallest share of all phenolic acids in the distillery stillage extracts (about 5–7%). In other studies on cereals and cereal products, ferulic, p-coumaric, and vanillic acids also made up the largest shares of the extracted acids (Kim et al., 2010). Ferulic and p-coumaric acids are covalently bound in the esterified or etherified form to polymers (lignin and hemicellulose) of the cell walls of cereals (Lu et al., 2021). In this form, their availability is low, but it can be assumed that the anaerobic treatment of the cereals releases ferulic and p-coumaric acids, while other acids can be degraded by fermentation.

3.2.2. Kinetics of extraction

The efficiency of recovery is affected by the type of raw material and intermolecular interactions, which may be synergistic or antagonistic, depending on the conditions and the compounds tested. Therefore, for each raw material, when optimizing the extraction conditions, kinetic modeling should be performed to explain the process. In the present study, for the first time, kinetic modeling to describe SWE from distillery stillage was successfully applied.

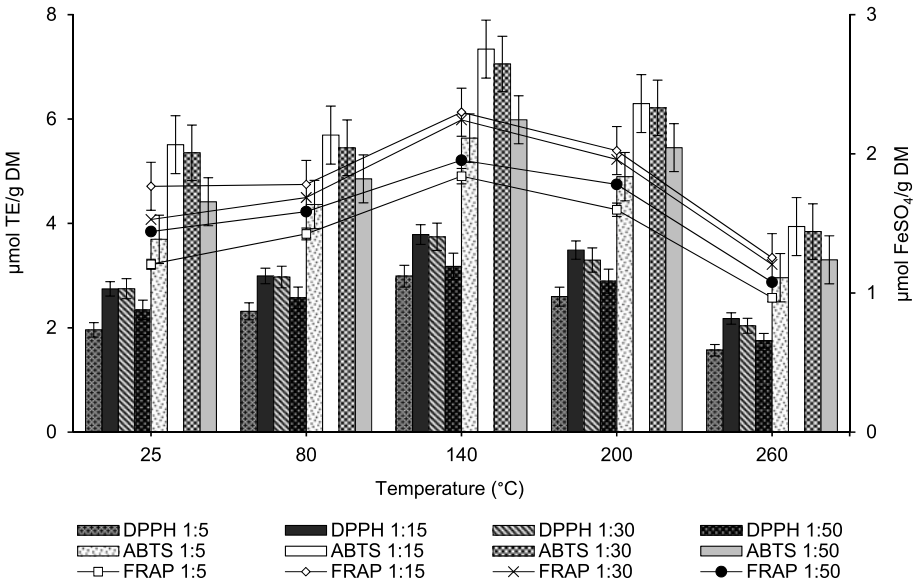


Fig. 4. Antioxidant activities of the distillery stillage extracts that were obtained with 30-min SWE.

A second-order kinetic model is suitable for explaining the effect of SWE time on the recovery of phenolic acids from distillery stillage. Table 1 shows the ERs, which were calculated for times of 0–30 min, which was the period in which the increase in the content of phenolic acids was observed. For all the experimental conditions, the coefficient of determination (R^2) was 0.97–0.99, indicating that the second-order kinetics equations fit the experimental results well.

The ERs for total and individual phenolic acids ranged from 0.450 to 1.333 µg/(g DM·min) and from 0.030 to 0.658 µg/(g DM·min), respectively. The highest values were achieved at 140 °C and an *r* value of 1:15 (w:v) (Table 1). ERs for p-OH benzoic, vanillic, syringic, and sinapic acids at the *r* values of 1:15 and 1:30 (w:v) were similar. However, the ERs for p-coumaric and ferulic acids were significantly higher (1.40- and 2.49-times, respectively) at the *r* value of 1:15 than at one of 1:30 (w:v). Moreover, as the temperature was increased from 25 to 140 °C, the ER increased (about 1.52–2.10-times), but a further increase to 260 °C decreased the ER. The ERs of phenolic acids were 1.17–1.70- and 1.45–2.82-times higher at 140 °C than at 200 and 260 °C, respectively. These results indicate that a properly selected temperature significantly improves the efficiency and profitability of phenolic acid recovery from distillery stillage. Water under subcritical conditions increases the solubility of antioxidant compounds, reducing the dielectric constant and thus increasing diffusion (Giombelli et al., 2020), which promotes the penetration of the solvent into the sample matrix and results in easier recovery of target compounds.

3.3. Antioxidant properties

The amount and composition of phenolic acids is a key determinant of the antioxidant activity of the extracts and depends on the recovery conditions. Therefore, optimal extraction conditions should increase the efficiency of phenolic acid recovery and the antioxidant activity of the extracts. However, as a result of synergy, components found in the extracts other than phenolic acids can significantly affect the antioxidant activity. Therefore, in the present study, the antioxidant activity of the extracts was monitored using the ABTS, DPPH, and FRAP assays to understand the relationship between the structure of the compounds and their activity. Fig. 4 shows changes in the antioxidant activity of polyphenols extracted by 30-min SWE (the most efficient extraction variant) depending on the temperature and the *r* value; the activities obtained with the other time variants are shown in Fig. 3SM.

Extracts obtained with 30-min SWE at 140 °C had the highest antioxidant activity (ABTS: 7.05–7.34 µmol TE/g DM; DPPH: 3.74–3.79 µmol TE/g DM; FRAP 2.24–2.30 µmol $FeSO_4$/g DM) (Fig. 4, Fig. 3SM). A further temperature increase to 260 °C significantly decreased the activity. The activities obtained with the ABTS, DPPH, and FRAP assays were 1.84-, 1.75-, and 1.86-times higher at 140 °C than at 260 °C, respectively. The extraction temperature significantly affected the antioxidant activity; from highest to lowest activity, these were the results obtained with the temperature variants: 140 °C > 200 °C > 80 °C > 25 °C > 260 °C. The activity depended on the amount of phenolic compounds recovered from the distillery stillage; the highest content of phenolic acids was also observed in the extracts obtained at 140 °C (Fig. 2). The antioxidant activities were strongly positively correlated with phenolic acid content (r_s=(0.94)–(0.96); $p \leq 0.05$) (Fig. 4SM). In the case of all three assays, the antioxidant activities of the extracts obtained at the *r* values of 1:30 and 1:15 were similar, independently of temperature. Despite the differences in antioxidant activity resulting from the variable extraction conditions, in all extracts, the ABTS assay showed a much higher antioxidant activity than the DPPH assay, which was followed by the FRAP assay (Fig. 4). These differences may have occurred because each test is based on a different reaction mechanism (ABTS based on HAT and DPPH and FRAP based on SET) (Liang and Kitts, 2014), the efficiency of recovery of the different types of antioxidants varies, and the antioxidants react differently with the radicals used. However, in the case of antioxidants from natural sources, these mechanisms can occur simultaneously and may be interrelated. In all distillery stillage extracts obtained in the present study, the ABTS, DPPH, and FRAP values were strongly positively correlated (r_s=(0.96)–(0.98); $p \leq 0.05$) (Fig. 4SM). The dominant antioxidant mechanism depends on the material from which the antioxidants are obtained, their structure and properties, as well as the recovery conditions (Awika et al., 2003). Therefore, it is important to demonstrate the effect of the molecular structure of phenolic acids on their antioxidant activity to gain fundamental insight into their free radical scavenging ability.

In the present study, hydroxycinnamic acids (ferulic, p-coumaric, and sinapic acids) accounted for about 68% of all free phenolic acids and predominated over hydroxybenzoic acids (p-OH benzoic, vanillic, syringic acids). Thus, hydroxycinnamic acids may be considered largely responsible for the antioxidant activity of the extracts from the distillery stillage. Similarly, Natella et al. (1999) reported that hydroxycinnamic acids had a stronger antioxidant activity than hydroxybenzoic acids. Both acids have a carboxylic group that has the ability to donate electrons, but the coupling and induction effects make COOH

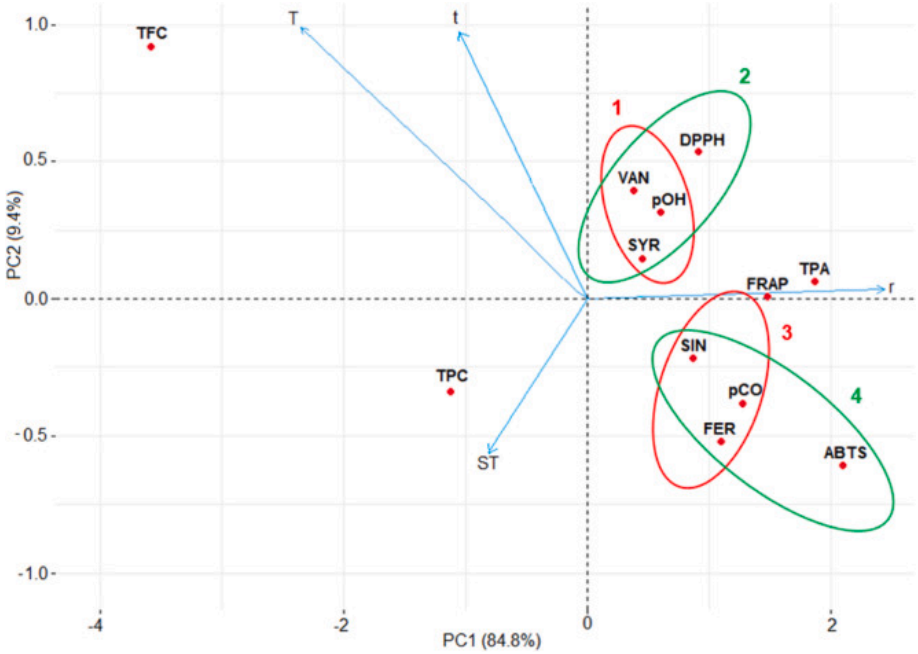


Fig. 5. PCA of distillery stillage extract characteristics, according to their phenolic profile and antioxidant activities under various recovery conditions. The red dots indicate the concentrations of compounds and their antioxidant activity. The blue arrows indicate the extract and extraction characteristics; t – extraction time, T – extraction temperature, r – solid-to-solvent ratio, ST – surface tension, pOH – p-OH benzoic acid, VAN – vanillic acid, SYR – syringic acid, pCO – p-coumaric acid, FER – ferulic acid, SIN – sinapic acid, TPA – total phenolic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(hydroxybenzoic acids) a strong electron-withdrawing group and –CH = CHCOOH (hydroxycinnamic acids) a weak electron-withdrawing group. Thus, in hydroxycinnamic acids, the –CH = CHCOOH group can increase the electron cloud density of the benzene ring, reduce the dissociation energy of phenolic hydroxyl bonds, and then increase the free radical scavenging ability (Chen et al., 2020). It is also worth noting that hydroxyl or methoxy groups linked to aromatic rings determine the bioactive properties of phenolic acids. The methoxy group stabilizes the interaction of the electron pair on the oxygen atom, thus increasing the ability to scavenge free radicals (Ammar et al., 2009). The hydroxyl group on the aromatic ring is an electron donor and thus reduces the dissociation energy of the hydroxyl bonds, which improves their free radical scavenging ability (Chen et al., 2020). The number and position of methoxy and hydroxyl groups affect the antioxidant activity of the acids (Shahidi and Zhong, 2015), thus affecting their usefulness in the food, pharmaceutical, and cosmetic industries.

3.4. Principal component analysis (PCA)

PCA was performed to show the relationship between the characteristics of the distillery stillage extracts in terms of polyphenol content and antioxidant activity and the variables related to SWE conditions. The analysis showed that the variables were reduced to two main components (PC1: 84.8%; PC2: 9.4%), which together accounted for 94.2% of the total variance of the studied variables (Fig. 5).

PC1 corresponded mainly to the content of individual phenolic acids and total phenolic acids (TPA), TPC; the values of the ABTS, DPPH, and FRAP assays, while PC2 corresponded to TFC. Variables located close to each other are correlated. Variables on the same side of the starting point (0,0) are positively correlated, and variables on the opposite side of the starting point are negatively correlated. Hence, all values for individual phenolic acids (i.e., VAN, pOH, SYR, SIN, pCO, and FER), TPA, antioxidant activity (ABTS, DPPH, and FRAP), and the *r* value were grouped in a cluster on the right side of the plot, which suggests that they are positively correlated. In the cluster on the left side of the plot, positively correlated variables are represented by TPC, TFC, temperature, extraction time, and ST. The variables in the cluster on the right side of the plot were negatively correlated with the variables from the cluster on the left side. In addition, PCA suggests that the content of phenolic acids, which depended on SWE conditions, had a large effect on the antioxidant activity of the obtained extracts. Therefore, it can be assumed that there were no other components of the extracts that could significantly affect the antioxidant activity. Thus, SWE allows high-quality extracts to be obtained from distillery stillage.

Additionally, using the PCA plot, it is possible to estimate the effect of each variable based on its distance from the starting point (Hardle and Simar, 2007). In the present study, phenolic acids were grouped into PC1; however, their effect on the antioxidant activity of the obtained extracts decreased in the following order: FER > pCO > VAN > SIN > pOH > SYR. This indicates that ferulic and p-coumaric acids had the greatest effect on the antioxidant activity of the extracts obtained. For the parameters describing SWE, the order of importance was as follows: T > t > r > ST. Thus, the extraction temperature and time had the greatest effect on the recovery of bioactive compounds (as described in section 3.1.3).

Moreover, the relationship between phenolic acids and their antioxidant activity obtained in 3 tests (ABTS, DPPH, and FRAP) was determined by employing Euclidean cluster analysis. The PCA plot contains 4 clusters: cluster 1 (hydroxybenzoic acids), cluster 2 (hydroxybenzoic acids and DPPH), cluster 3 (hydroxycinnamic acids), cluster 4 (hydroxycinnamic acids and ABTS). This reflects the differences in the structure of phenolic acids and their association with the antioxidant activity of the extracts. As can be seen, cluster 3 is further from the starting point than cluster 1, indicating that hydroxycinnamic acids had a stronger association with the antioxidant activity of the extracts than did hydroxybenzoic acids. In the present study, the antioxidant activity depended on the amount of phenolic acids recovered: due to the predominance of hydroxycinnamic acids, ABTS values were highest (cluster 4), whereas, because of the lesser amount of hydroxybenzoic acids, DPPH values were lower (cluster 2). In the present study, the PCA indicated that, to obtain high-quality extracts *via* SWE of distillery stillage, the greatest attention should be paid to monitoring the temperature and time of extraction.

3.5. Characteristics of the biomass remaining after SWE

In the present study, the composition of the biomass remaining after SWE of polyphenols was examined to evaluate whether the temperature-induced changes were sufficient to consider the biomass a hydrochar. Hydrothermal carbonization of biomass takes place when water is used

Table 2
Compositional of elements and energy value of solid residue after SWE.

Type of sample	ODM (%)	%C	%H	%N	%S	%O	atomic H/C ratio	atomic O/C ratio	HHV (MJ/kg ODM)	HHV improvement (%)	Energy desinfication
distillery stillage	95.89	52.18	7.63	6.18	0.35	29.55	1.75	0.42	23.35		
solid residue SWE 25	98.92	56.71	8.32	7.07	0.45	26.38	1.76	0.35	26.45	13.27	1.13
solid residue SWE 80	98.13	57.23	8.32	7.18	0.42	24.99	1.74	0.33	26.86	15.03	1.15
solid residue SWE 140	97.94	57.69	8.36	6.89	0.43	24.58	1.74	0.32	27.14	16.24	1.16
solid residue SWE 200	97.57	59.40	8.55	6.27	0.39	22.96	1.73	0.29	28.26	21.04	1.21
solid residue SWE 260	95.25	63.71	8.95	4.64	0.18	17.78	1.69	0.21	31.14	33.38	1.33

Note: ODM – organic dry matter; HHV – higher heating value.

as a solvent in combination with elevated temperatures (150–350 °C) and pressures (2–6 MPa), (Mäkelä et al., 2015; Titirici et al., 2012; Arellano et al., 2016). An increase in the carbon content and decreases in the oxygen and hydrogen contents indicate the conversion of the biomass into a solid product termed hydrochar. Hydrochar can be used as solid fuel, soil amendment, or a precursor of activated carbon (Cao et al., 2021). In the present study, with an increase in the process temperature, the carbon content of the remaining biomass increased, and its oxygen content decreased; thus, biomass derived at 260 °C had the highest carbon content (63.71%) and the lowest oxygen content (17.78%) (Table 2). This was the result of carbonization and gasification reactions that are favored by temperatures above 200 °C (Watson et al., 2020). In the present study, the atomic H/C and O/C ratios of the biomass remaining after SWE at 260 °C were lower than those of untreated stillage by 4% and 50%, respectively. These chemical changes are due to the removal of hydroxyl groups *via* dehydration, the removal of carboxyl and carbonyl groups *via* decarboxylation, and the cleavage of many ester and ether bonds *via* hydrolysis (Zhang et al., 2019a). The contents of nitrogen (4.64%) and sulfur (0.18%) in the biomass derived at 260 °C were significantly lower than those in the untreated biomass (by 6.18% and 0.35%, respectively) because the nitrogen and sulfur oxides formed during the carbonization process were dissolved in hot water (Hoekman et al., 2013). Due to improved carbonization with increased temperatures, the estimated HHV increased from 23.35 MJ/kg ODM in the untreated stillage to 31.14 MJ/kg ODM in the 260°C-derived biomass. In other words, the highest temperature improved the HHV by 33%. The temperature-induced increase in the energy densification ratio up to 1.33 is mainly attributable to changes in the O/C ratio (Deng et al., 2022). Thus, the present study indicates that the biomass remaining after SWE of polyphenols from distillery stillage can be considered a valuable product. However, before its further utilization as a solid fuel or activated carbon precursor, its heating value should be measured, and its FT-IR spectra should be determined.

4. Conclusions and future directions

The results obtained in the present study show that SWE effectively recovers bioactive compounds from the distillery stillage and that water under subcritical conditions (temperature of 140 °C and pressure of 4.14 MPa) can be used as a solvent. This provides the opportunity to develop green extraction techniques for recovering bioactive polyphenols from different wastes, including distillery stillage. RSM and PCA both indicated that temperature and extraction time had a large effect on the recovery yield and antioxidant activity of the obtained extracts and that the solid-to-solvent ratio had a lesser effect. With 30-min SWE at a solid-to-solvent ratio of 1:15 (w:v), the highest TPC (4.88 ± 0.16 mg GAE/g DM) and TFC (1.24 ± 0.23 mg QUE/g DM) were obtained at 140 °C and 200 °C, respectively. With increases in the extraction time to 90 min and the temperature to 260 °C, a significant decrease in TPC (about 4.4-times) and TFC (about 3.9-times) was observed. Phenolic acids were present in the extracts mainly in free forms (up to 88% of the total content), which allows the hydrolysis step to be omitted, thus avoiding the unnecessary use of toxic reagents. The antioxidant activity correlated positively with the content of individual phenolic acids in the extracts. In all extracts, the measured antioxidant activity was highest with the ABTS assay, followed by the DPPH assay, and then the FRAP assay. Hydroxycinnamic acids (especially ferulic and p-coumaric acids) were mostly responsible for the antioxidant effect of the extracts. The results indicate that the recovery of phenolic acids from distillery stillage can increase the economic profitability of distilleries and the rate of by-product utilization while reducing the amount of environmental pollutants, which is commensurate with the principles of a circular economy. Therefore, this study constitutes an important contribution to the literature, considering the lack of data on the extraction of bioactive compounds from distillery stillage under subcritical conditions. In this extraction process, the elimination of the organic solvent and the resulting solvent residues in the extracts makes purification of the extracts easier and is desirable to produce organic functional food products and nutraceuticals. Therefore, SWE can be considered a cost-effective and environmentally-friendly process for extracting antioxidants from biomass. The estimated heating value of the biomass remaining after SWE at 260 °C was 33% higher than that of the untreated distillery stillage because of temperature-induced carbonization and reduction of O/C ratio, which increases the range of applications of distillery stillage. Taking into account that polyphenol extraction was the most effective at 140 °C and the heating value of the remaining biomass was the highest at 260 °C, the process temperature should be chosen depending on the desired output.

Author contributions

Wioleta Mikucka: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft Preparation, Visualization. **Magdalena Zielinska:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft Preparation, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Katarzyna Bulkowska:** Methodology, Validation, Writing – review & editing, Supervision. **Izabela Witonska:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.115548.

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Subcritical water extraction of bioactive phenolic compounds from distillery stillage

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Supplementary Material

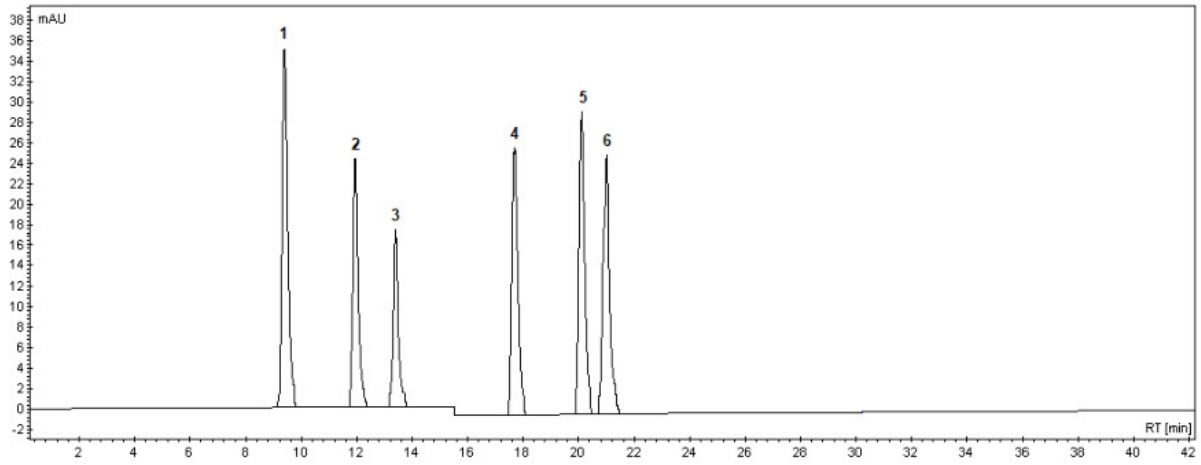


Fig. 1SM. HPLC chromatogram of phenolic acid standards at 260 nm (peaks: (1) p-OH benzoic acid; (2) vanillic acid; and (3) syringic acid) and 320 nm (peaks: (4) p-coumaric acids; (5) ferulic acid; and (6) sinapic acid)

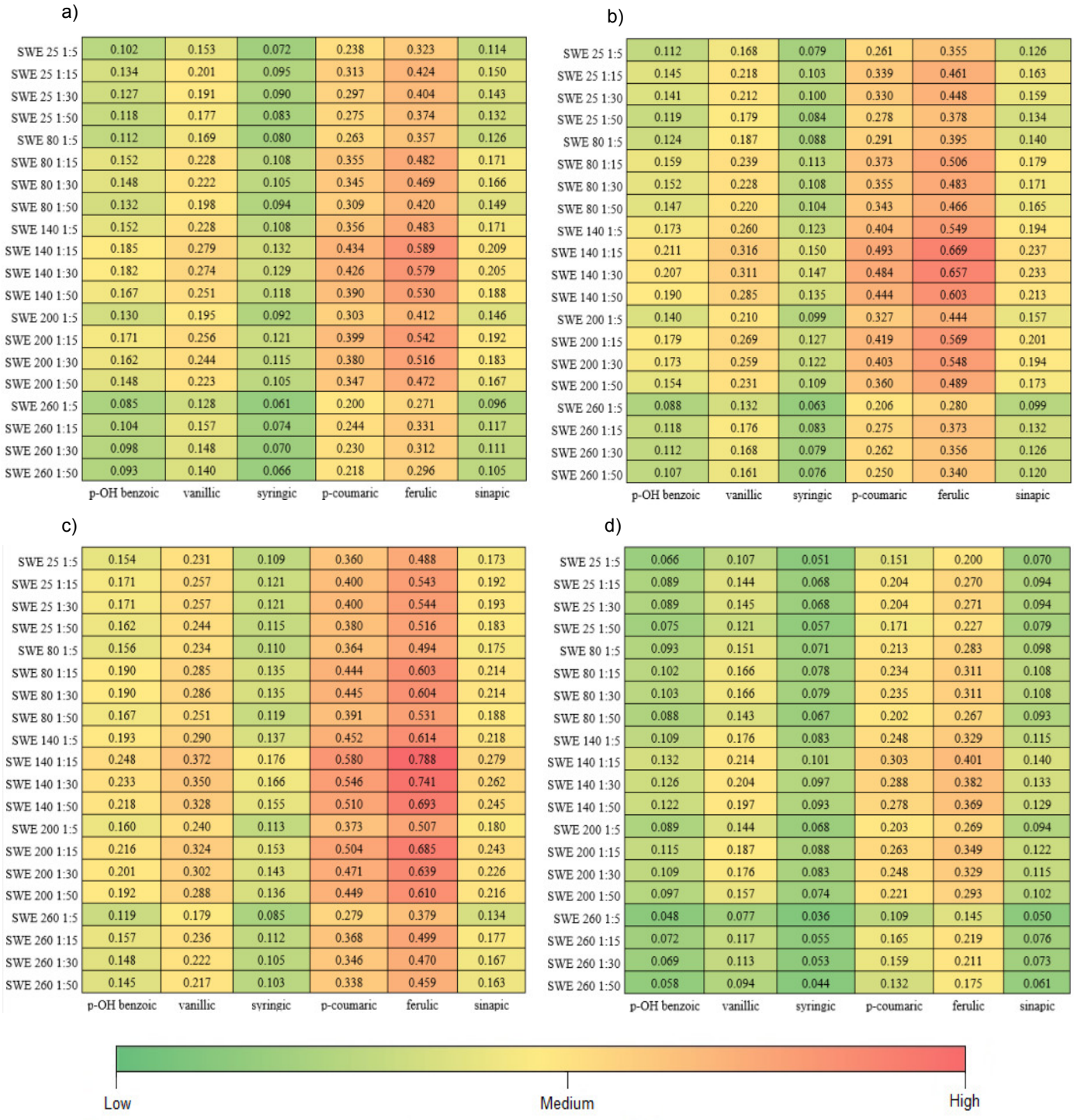


Fig. 2SM. Heatmap of the contents of individual phenolic acids (µg/g DM) in distillery stillage extracts obtained with (a) 5-min, (b) 15-min, (c) 60-min and (d) 90-min SWE. In the abbreviations, the values after SWE indicate the extraction temperature followed by the *r* value. The content of individual phenolic acids is represented by a color (red indicates a high content; green indicates a low content)

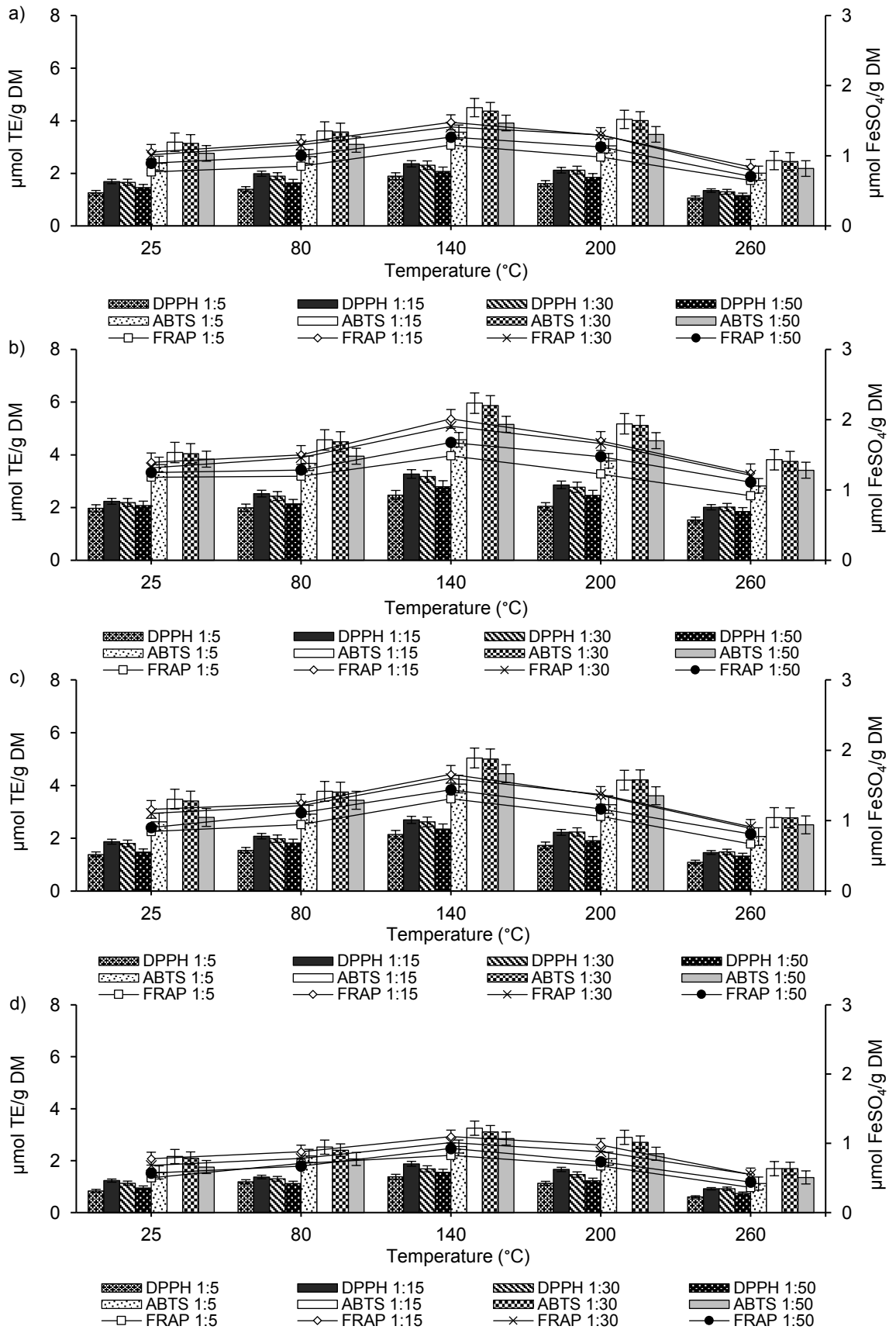


Fig. 3SM. Antioxidant activities of the distillery stillage extracts that were obtained with (a) 5-min, (b) 15-min, (c) 60-min and (d) 90-min SWE

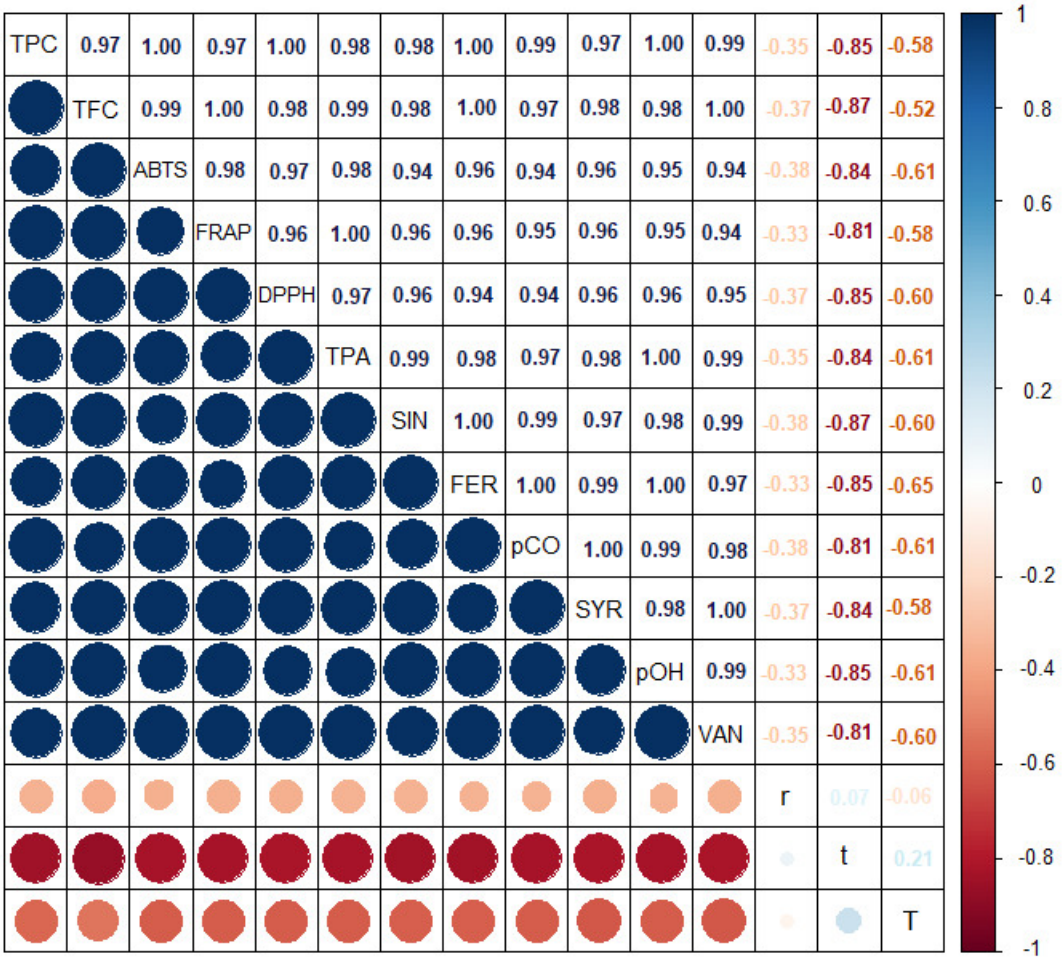


Fig. 4SM. Correlations between TFC, TPC, concentrations of phenolic acids, ABTS, FRAP, and DPPH and SWE conditions. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circles are proportional to the correlation coefficients. Values of Spearman's rank correlation coefficient above 0.6 are described as a strong correlation; t – extraction time, T – extraction temperature, r – solid-to-solvent ratio, pOH – p-OH benzoic acid, VAN – vanillic acid, SYR – syringic acid, pCO – p-coumaric acid, FER – ferulic acid, SIN – sinapic acid, TPA – total phenolic acid

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Attachment 6.

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Individual Phenolic Acids in Distillery Stillage Inhibit Its Biomethanization

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Abstract: Polyphenols that are abundant in various organic wastes can inhibit anaerobic degradation of these wastes. This study investigated the effect of the concentration of individual phenolic acids (p-OH benzoic, vanillic, ferulic, sinapic, syringic, and *p*-coumaric acids) and their mixture on the methane potential of distillery stillage. An increase in phenolic acid concentration adversely affected biogas production and composition, as well as the methane-production rate. The inhibition constants for methane production were 0.5–1.0 g/L of individual phenolic acids and 1.5 g/L of the mixture of these acids. At lower concentrations, the phenolic acids were utilized as a carbon source, but the process was impeded when their concentrations exceeded the threshold value, due to their negative effect on microbial growth. When distillery stillage was spiked with vanillic acid, two-phase methane production was observed. Spiking distillery stillage with vanillic, *p*-coumaric, syringic, or ferulic acids affected anaerobic digestion the most; 2 g/L of these acids completely inhibited methane production. With 4.0 g/L of all individual phenolic acids, no methane production was observed. As the concentration of these phenolic acids increased from 0.5 to 4.0 g/L, the abundance of methanogenic Archaea, in which acetoclastic methanogens predominated, decreased by about 30 times.

Keywords: anaerobic digestion; distillery stillage; methanogenesis; FISH; PCA

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1. Introduction

To achieve sustainable ecological and economic development, renewable energy sources need to be investigated, e.g., by managing waste to produce biogas. Among the substrates for biogas production, by-products from distilleries (distillery stillage) are used because of their widespread availability and high organic content. Annually, 80 million tons of stillage are produced around the world, which causes significant environmental pollution due to its high concentrations of difficult-to-degrade organics and nitrogen, its low pH, and its dark brown color [1]. To utilize this waste, anaerobic processing is commonly used, as the resulting power generation can significantly reduce operating costs when compared to energy-intensive aerobic processing [2].

However, distillery waste requires treatment after bio-methanization and before its potential use as fertilizer, due to its high residual COD, low biodegradability index, and toxicity [3]. Its toxicity is partially due to the content of polyphenols in it [4,5], which are not readily biodegradable and have significant antimicrobial activity [3,5]. Polyphenols reduce the ability of methanogens to produce methane; at phenolic compound concentrations from 120 to 594 mg/L, biogas production was reduced by up to 50% [6]. When treating olive mill wastewater, a polyphenol concentration of 9 g/L significantly limited anaerobic digestion [7]. On the other hand, a polyphenol content of 2.1–21.4 g/kg total solids did not affect the biological methane potential of grape pomace [8]. This could have been related to the fact that anaerobic microorganisms have some degree of tolerance to phenols, and low concentrations may favor anaerobic digestion [9]. It should also be considered that biomass

pretreatment causes the solubilization of polyphenols as well as their release [4]. Therefore, to promote biomethane production and to ensure the profitability of biogas plants, methods for accelerating biomethane production from waste containing polyphenols should be further investigated.

Interestingly, however, the presence of polyphenols in distillery stillage can be seen in a positive light, due to the useful properties of these aromatic compounds. They are found not only in stillage, but also in olive-mill waste, wheat straw, fruit and vegetable waste, and wastewater from winemaking [10–13]. Due to their strong antioxidant activity, they have received much attention as valuable bioactive compounds of commercial interest. As a result, the recovery of polyphenols from waste has started to be investigated with the aim of utilizing them in cosmetics, pharmaceuticals, and food industry products [12].

The quality and quantity of polyphenols in different wastes can vary, depending on the type of plant material from which the waste was generated, the processing technology, the storage conditions, and other factors such as pH, oxygen, temperature, light, metal ion enzymes, etc. [14]. Regarding distillery waste, only the effect on the bio-methanization of complete polyphenols from sugarcane molasses distillery wastewater has been investigated [15]. Polyphenols include phenolic acids and flavonoids. Phenolic acids differ in their activity, concentrations, and proportions depending on the waste type [12]. Depending on the waste type, phenolic acids are present as lignin-bound compounds or in free forms, which determines the method of their recovery. In distillery stillage coming from cereal processing, their free forms predominate [16]. The effects of specific phenolics on bio-methanization were investigated only for brown seaweed [17]; however, in this study, phlorotannin was investigated, which is not a phenolic compound typically found in distillery stillage. Hence, based on literature reports, it is impossible to conclude if bio-methanization is adversely affected by total polyphenols or by specific phenolic acids; thus, it is difficult to decide which method of polyphenol recovery from particular waste should be performed before its anaerobic processing.

Although phenolic acids differ in their antimicrobial activity, depending on their structure, specific information about the effect of the particular acids present in distillery stillage on its bio-methanization potential is lacking. Therefore, the novelty of the study was to investigate the effects on bio-methanization of six phenolic acids (p-OH benzoic, vanillic, ferulic, sinapic, syringic, and *p*-coumaric acids) representative of those present in distillery stillage generated during alcohol production from cereals. The objectives of the study were to assess the effect of the type and concentration of the individual phenolic acids and their mixtures on (i) the biogas potential of the distillery stillage, (ii) the methane content in the biogas, (iii) the methane production rate, and (iv) the abundance of methanogenic Archaea in the microbial communities of the stillage. The technological and molecular results provide a comprehensive picture of the extent to which phenolic acids inhibit the growth of the microorganisms participating in the bio-methanization of the stillage and affect methane production. These results will help to decide if polyphenols should be recovered prior to stillage fermentation.

2. Materials and Methods

2.1. Characteristics of Substrate

The substrate for methane fermentation was distillery stillage (Table 1) collected from a company located in northeast Poland that produces concentrated unpurified ethyl alcohol from cereals. The total polyphenol content (TPC) in the stillage was 4.56 ± 0.03 mg gallic acid equivalent/g dry mass. The stillage was stored at 6 °C. As an inoculum, anaerobic sludge from the pilot-scale agricultural biogas plant processing cattle slurry and maize silage was used (organic loading rate (OLR) of 2.0 kg VS/(m^3·d), retention time of 20 days, 35 °C). To ensure degradation of easily degradable organic matter and to remove dissolved methane, the inoculum collected from the biogas plant was stored with an anaerobic headspace for 3 days at 37 °C.

Table 1. Characteristics of distillery stillage and inoculum.

Characteristic	Unit	Distillery Stillage	Inoculum
Total solids (TS)	g/kg	79.9 ± 3.0	61.5 ± 2.9
Ash	g/kg	3.7 ± 0.4	6.1 ± 1.4
Volatile solids (VS)	g/kg	76.2 ± 2.4	55.4 ± 1.9
Organics content	%	95.4	90.1
pH	-	6.52 ± 0.04	7.21 ± 0.01
Total COD	mg/L	109,000 ± 11,554	n.m.
Soluble COD	mg/L	43,600 ± 4830	8700 ± 347
Volatile fatty acids (VFAs)	mg/L	789.7 ± 0.8	571.2 ± 26.1
Ammonium nitrogen	mg/L	293.8 ± 4.7	162.4 ± 2.5
Total phosphorus (TP)	mg/L	212.4 ± 0.6	331.6 ± 2.0
Alkalinity	meq/L	182.8 ± 1.4	225.2 ± 1.1

n.m.—not measured.

2.2. Experimental Design for Methane Fermentation

The feedstock for the methane fermentation was a mixture of distillery stillage and inoculum (9.6:90.4, w:w) and was characterized by: 65.9 ± 2.4 g TS/kg, 51.2 ± 1.6 g VS/kg, 23,600 ± 689 mg COD_{sol}/L, 1047.8 ± 149.3 mg VFA/L, 152.6 ± 4.1 mg N-NH_4/L, and 116.6 ± 1.2 mg TP/L. The densities of the distillery stillage and inoculum were 1031 and 1014 kg/m^3, respectively. In series 1, the effect of individual phenolic acids (p-OH benzoic, vanillic, ferulic, sinapic, syringic, and *p*-coumaric acids) on bio-methanization was investigated. Individual phenolic acids were added to the feedstock for final phenolic acid concentrations of 0.5, 1.0, 1.5, 2.0, and 4.0 g/L. In series 2, to investigate the effect of a mixture of phenolic acids on bio-methanization, the same phenolic acids were mixed in equal proportions and added to the feedstock to obtain the final total phenolic acid concentrations in the mixture of 0.5, 1.0, 1.5, 2.0, and 4.0 g/L.

The methane production was measured in an automatic methane potential batch-test system (AMPTS II Bioprocess Control, Lund, Sweden). In this system, i.e., in 0.5 L reactors, the volume of the mixture of the inoculum and stillage was 100 mL, which ensured an initial VS content of 5 g/L. The reactors were equipped with a mixing system (100 rpm for 30 s every 10 min). CO_2 traps were used. To ensure anaerobic conditions, the samples were purged with nitrogen gas. The incubation time of the feedstock was 21 days at 37 °C. As a control, a sample of feedstock without phenolic acid addition was used. The tests were made in triplicate. Methane production was expressed as volume (L) at normal conditions (temperature and pressure) per kg of VS added initially. Based on methane production obtained from this measurement and the composition of the biogas, biogas production was calculated. Simultaneously, the methane production from the inoculum was measured. To take into account the inoculum's background and represent the methane potential of the distillery stillage accurately, the methane production from the inoculum was subtracted from the methane production from the feedstock [18]. The maximum theoretical methane potential of the substrate was calculated based on the assumption that 0.35 L of methane can be produced from 1 g of total COD [19].

2.3. Analytical Methods

In samples of stillage before and after fermentation (digestate), the contents of TS, VS, total COD, soluble COD, ammonium nitrogen, VFAs, and alkalinity were measured according to APHA [20], and the TP concentrations were measured with cuvette tests (Hach Lange GMBH, Berlin, Germany). For pH measurement, a pH meter (HANNA Instruments HI 221, Cluji-Napoca, Romania) was used. Total organic carbon (TOC) and dissolved organic carbon (DOC) concentrations were measured with a Fusion analyzer (Teledyne Tekmar, Mason, OH, USA). The concentrations of soluble COD, ammonium nitrogen, VFAs (expressed as acetic acid), TP, alkalinity, and pH were measured in the supernatants obtained after the centrifugation of the distillery stillage (10 min, 8000 rpm). Free ammonia

was calculated according to Capson-Tojo et al. [21]. Before and after fermentation, the TPC was determined using the Folin-Ciocalteu method [22].

The biogas composition was assessed by injecting 20 mL gas samples, collected from the headspace of the reactors, into a gas chromatograph (GC, 7890A Agilent, Santa Clara, CA, USA) with a thermal conductivity detector (TCD) operating at 250 °C. Two Hayesep Q columns (80/100 mesh), two columns with molecular sieves (60/80 mesh), and a Porapak Q column (80/100) were used to separate the biogas components. Carrier gases (helium and argon) were used as the mobile phase at a flow rate of 15 mL/min.

2.4. Abundance of Methanogenic Microorganisms

Biomass samples (three replicates collected after fermentation from digestates) were fixed and subjected to fluorescence in in situ hybridization (FISH) [23]. The following molecular probes were used: ARC915 (Archaea; [24]), MSMX860 (Methanosarcinaceae; [25]), MX825 (*Methanosaeta*; [25]), and MB1174 (Methanobacteriaceae; [25]) under the conditions given in ProbeBase (www.microbial-ecology.net/probebase, accessed on 17 September 2020). The samples were visualized with an epifluorescence microscope (Nikon Eclipse 50I, Tokyo, Japan). To quantify the FISH-defined populations, ImageJ software (http://rsb.info.nih.gov/ij/, accessed on 13 September 2020) was employed; at least 30 fields of view were examined for each probe. To determine the bio-area of the populations in the sample, the ratio of cells targeted by each probe to those stained with DAPI (4,6-diamidino-2-phenylindole) was calculated and expressed as the relative abundance (as a percentage) of all detected microorganisms. The coefficients of variation of all values were 10–15%.

2.5. Data Analysis

First order kinetics was the assumed model for methane production:

$$C_t = C_0 \cdot \left(1 - e^{-k \cdot t}\right),$$

where C_t (L CH_4/kg VS) was the cumulative methane production at digestion time t (days), C_0 (L CH_4/kg VS) was the maximal methane production, and k (1/d) was the constant of methane production. C_0 and k were estimated using nonlinear regression and the Gauss-Newton algorithm (Statistica 13.1). The rate of methane production (r) was calculated as $r = k \cdot C_0$. In the case of a two-phase course of methane production, the r values were calculated for each phase separately. The goodness of fit of the model to the data was evaluated using the determination coefficient (R^2).

Spearman's rank correlation and Principal Component Analysis (PCA) were calculated to determine the relationships between the operating conditions, biogas/methane production from stillage spiked with various phenolic acids, and microbial abundance using RStudio Version 1.2.1335. The matrix of correlations was visualized with a correlogram using the "corrplot" package. The relationships between the variables were determined using the correlation coefficient (r_s). PCA was plotted with the "ggplot2" and "ggfortify" packages. Heat maps show the effect of phenolic acid concentrations on the biogas composition and a distribution of the abundance of Archaea and potential methanogens.

In all analyses, the statistical significance was determined at the 95% confidence interval.

3. Results and Discussion

3.1. Methane Production

The addition of phenolic acids to distillery stillage diminished the methane production, and this decrease depended on the type and concentration of the phenolic acids. The cumulative methane production at the end of the incubation period is shown in Figure 1. Biogas production per kg of VS and kg of fresh mass is given in Table S1.

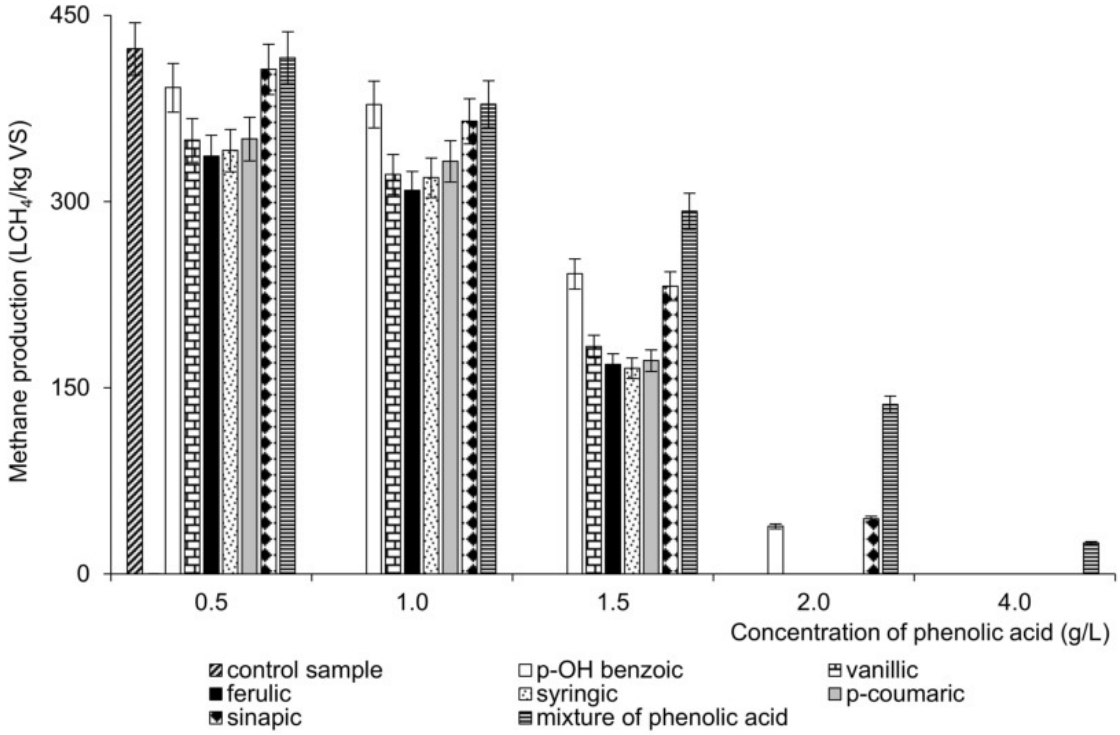


Figure 1. Cumulative methane production at the end of the experiments.

The anaerobic processing of raw distillery stillage not spiked with phenolic acids (control sample) produced 423 L methane/kg VS after 21 days (Figure 1). Similarly, methane yields were 410, 401–458, 389.1, and 503 L/kg VS after the 30-day incubation of whole-cereal stillage [26], 22-day incubation of whole-corn stillage [27], 30-day incubation of barley stillage [28], and 41-day incubation of barley stillage [29], respectively. In series 1, with concentrations of individual phenolic acids of 0.5 and 1.0 g/L, slightly lower amounts of methane were produced: from 309 L/kg VS with ferulic acid to 406 L/kg VS with sinapic acid. Increasing the phenolic acid concentration significantly decreased methane production. With 1.5 g/L of phenolic acids, the respective amounts of methane produced were 43–61% lower than the amounts in the control sample, depending on the phenolic acid that was used. With 2.0 g/L of p-OH benzoic and sinapic acids, the methane production accounted for 9 and 10% of that measured for the control sample. The acids that had the strongest effects on methane production were vanillic, ferulic, syringic, and *p*-coumaric acids. Their presence at 2.0 g/L resulted in no methane production. With 4.0 g/L of all acids present individually, methane was not produced.

Changing the phenolic acid concentrations in the distillery stillage changed the average shares of methane and carbon dioxide in the biogas (Figure 2). In series 1, at phenolic acid concentrations of 0.5 and 1.0 g/L, the methane percentage was similar to that in the control sample: from 71.85% for *p*-coumaric acid to 76.78% for p-OH benzoic acid. At phenolic acid concentrations of 1.5 and 2.0 g/L (for p-OH benzoic and sinapic acids), the methane contribution was lower by 17–25 and 28–30%, respectively, than in the control sample.

In series 2, phenolic acid mixtures at concentrations of 0.5–1.5 g/L had little effect on methane production, whereas mixtures at concentrations of 2.0 and 4.0 g/L reduced methane production by 68 and 94%, respectively, compared to the control sample (Figure 1). The differences in methane and carbon dioxide content were not as marked as in series 1 (Figure 2). At concentrations of 1.5 and 2.0 g/L, the methane content decreased by 12 and 19%, respectively, compared to the control sample. However, at 4.0 g/L, the methane content substantially decreased, and its share was 51.41%. At a given total concentration of phenolic acids, the inhibition of methane production was less with mixtures of the acids than with individual acids. This was particularly visible at 2.0 and 4.0 g/L in mixtures,

when methane was still produced. It can be explained by the fact that the concentrations of vanillic, ferulic, syringic, and *p*-coumaric acids, which inhibited methane production the most, were lower in mixtures than when these acids were present individually. The lower the proportion of these individual phenolic acids in the mixtures, the less they affected methane production. Additionally, the presence of phenolic acids in a mixture can cause them to polymerize or become insoluble. This insolubility may reduce interactions between the compound and cells and thus reduce their inhibitory capacities [30].

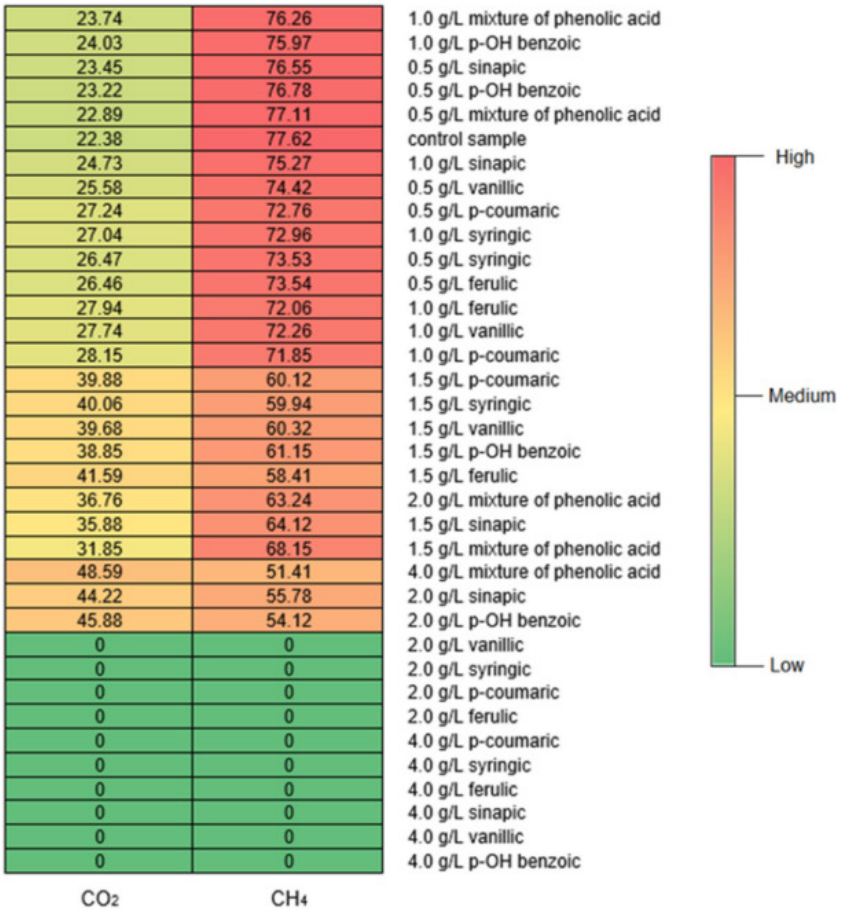


Figure 2. Percentage of methane and carbon dioxide in the biogas. Grouping was based on hierarchical agglomerative clustering. The percentage of methane and carbon dioxide is represented by a color (light blue means lower value; dark blue means higher value). The color key is indicated in the legend in the top left of the plot, along with the count of the same case in each titer.

The fact that low concentrations of phenolic acids (both individually and in the mixture) had little or no inhibitory effect on biogas and methane production indicates that they could have been converted into methane, as shown by Chapleur et al. [31] during the degradation of phenol-enriched biomass. They reported that concentrations of phenolic compounds above 1 g/L reduced the production of biogas from household waste by 10%, and a further increase in their concentration to 4.0 g/L reduced production by 98%. At phenol concentrations higher than 1 g/L, Dong et al. [9] reported a 50% decrease in methane yield. On the other hand, 0.5 g/L of polyphenols was identified as a concentration that inhibited the anaerobic digestion of olive oil mill wastewater because of a lack of microbial adaptation [32].

The maximum theoretical methane potential of the distillery stillage was 487 L/kg VS. A similar theoretical potential (479 L/kg VS) was obtained for cereal-processing stillage [33], which resulted from the total COD/VS ratio of 1.47 g/g (1.43 g/g in the present study, Table 1). This may be related to the high contribution of proteins (58.5% of dry matter) in

such wastes, in comparison with carbohydrates (9.74%) that are converted into alcohol and lipids (8.49%) [34]. In the present study, during the digestion of distillery stillage without phenolic acids, 86.8% of the theoretical methane potential was achieved. The effects of phenolic acids on methane production from distillery stillage as a percentage of the maximum theoretical methane production are given in Table 2. The percentage decreased to about 70% for 0.5 g/L of acids that affected the methane production the most (i.e., vanillic, ferulic, syringic and *p*-coumaric). At low concentrations of p-OH benzoic and sinapic acids, the methane production was maintained at a high level of about 80% of the maximum. During anaerobic incubation, after the first period of degradation of easily degradable compounds, there was a period in which the substrate became liquefied, which may have resulted in a release of slowly biodegradable compounds that became available to the microorganisms. This could have increased the methane production after several days of digestion. A similar observation was made by Battimelli et al. [35] during the anaerobic biodegradation of slaughterhouse waste in fed-batch reactors. This was explained by the fact that slowly biodegradable compounds were responsible for biogas production, in addition to the biogas produced by the degradation of the substrate, resulting in high biogas potential.

Table 2. Methane potential of distillery stillage spiked with phenolic acids as a percentage of the maximum theoretical methane potential.

Type of Sample	Percentage of Maximum Theoretical Methane Potential	Type of Sample	Percentage of Maximum Theoretical Methane Potential
control sample	86.8		
0.5 g/L p-OH benzoic	80.4	0.5 g/L ferulic	69.1
1 g/L p-OH benzoic	77.7	1 g/L ferulic	63.4
1.5 g/L p-OH benzoic	49.6	1.5 g/L ferulic	34.7
2 g/L p-OH benzoic	7.9	2 g/L ferulic	0.0
4 g/L p-OH benzoic	0.0	4 g/L ferulic	0.0
0.5 g/L vanillic	71.7	0.5 g/L syringic	70.0
1 g/L vanillic	66.1	1 g/L syringic	65.5
1.5 g/L vanillic	37.6	1.5 g/L syringic	34.0
2 g/L vanillic	0.0	2 g/L syringic	0.0
4 g/L vanillic	0.0	4 g/L syringic	0.0
0.5 g/L *p*-coumaric	72.0	0.5 g/L sinapic	83.5
1 g/L *p*-coumaric	68.2	1 g/L sinapic	74.9
1.5 g/L *p*-coumaric	35.3	1.5 g/L sinapic	47.6
2 g/L *p*-coumaric	0.0	2 g/L sinapic	9.1
4 g/L *p*-coumaric	0.0	4 g/L sinapic	0.0
0.5 g/L mixture	85.4		
1 g/L mixture	77.7		
1.5 g/L mixture	60.0		
2 g/L mixture	28.0		
4 g/L mixture	5.1		

The inhibition of methane fermentation by phenolic acids was also reflected in the kinetics of methane production, as can be observed over the course of methane production over time. In series 1 (0.5–1.5 g/L) (Figure S1a,c–f) and series 2 (0.5–2.0 g/L) (Figure S1g), the methane production increased until the 6th day of measurement, then the curves plateaued. At 2 g/L of p-OH benzoic and sinapic acids (Figure S1a,f) and at 4 g/L of the acid mixture (Figure S1g), a lag phase of several days was seen. In the case of the 0.5–1.5 g/L vanillic acid additive, methane production was divided into two phases (Figure S1b); it stabilized after the 3rd day, while from the 9th day it started to increase and reached a plateau after the 13th day of fermentation. This division of methane production

into two phases could have been caused by the fact that vanillic acid can only be utilized by microorganisms that need more time to grow (acetoclastic methanogens) [36]. Additionally, vanillic acid is not completely broken down by methane fermentation microorganisms. Only after vanillic acid was oxidized to ferulic acid could the next phase of methane fermentation take place [37]. The constants of methane production (k) were 0.11–1.38 1/d (Table 3). It varied because of the biological effects of toxicants and ranged from 0.08 to 0.09 l/d [38] or from 0.05 to 0.13 1/d [39] for organic waste biomass.

Table 3. Parameters of methane production using first-order kinetics.

Type of Sample	k (1/d)	R^2	C_0 (L/kg VS)	Type of Sample	k (1/d)	R^2	C_0 (L/kg VS)
control sample	0.52 ± 0.03	0.99	423 ± 11				
0.5 g/L p-OH benzoic	0.52 ± 0.02	0.99	392 ± 9	0.5 g/L ferulic	0.40 ± 0.01	0.99	337 ± 10
1 g/L p-OH benzoic	0.50 ± 0.02	0.99	378 ± 10	1 g/L ferulic	0.34 ± 0.02	0.99	309 ± 8
1.5 g/L p-OH benzoic	0.46 ± 0.01	0.99	242 ± 6	1.5 g/L ferulic	0.35 ± 0.01	0.98	169 ± 6
2 g/L p-OH benzoic	0.13 ± 0.01	0.99	38 ± 1	2 g/L ferulic	0	0	0
4 g/L p-OH benzoic	0	0	0	4 g/L ferulic	0	0	0
0.5 g/L vanillic phase I	0.98 ± 0.04	0.99	148 ± 7	0.5 g/L syringic	0.41 ± 0.02	0.99	341 ± 7
1 g/L vanillic phase I	0.72 ± 0.03	0.98	131 ± 4	1 g/L syringic	0.26 ± 0.01	0.99	319 ± 8
1.5 g/L vanillic phase I	1.38 ± 0.04	0.98	58 ± 2	1.5 g/L syringic	0.33 ± 0.01	0.99	166 ± 4
2 g/L vanillic phase I	0	0	0	2 g/L syringic	0	0	0
4 g/L vanillic phase I	0	0	0	4 g/L syringic	0	0	0
0.5 g/L vanillic phase II	0.13 ± 0.01	0.98	201 ± 9	0.5 g/L *p*-coumaric	0.52 ± 0.03	0.99	350 ± 9
1 g/L vanillic phase II	0.11 ± 0.01	0.98	191 ± 7	1 g/L *p*-coumaric	0.43 ± 0.02	0.99	332 ± 7
1.5 g/L vanillic phase II	0.19 ± 0.01	0.98	125 ± 5	1.5 g/L *p*-coumaric	0.33 ± 0.02	0.99	172 ± 7
2 g/L vanillic phase II	0	0	0	2 g/L *p*-coumaric	0	0	0
4 g/L vanillic phase II	0	0	0	4 g/L *p*-coumaric	0	0	0
0.5 g/L sinapic	0.51 ± 0.02	0.99	407 ± 10	0.5 g/L mixture	0.41 ± 0.02	0.99	416 ± 13
1 g/L sinapic	0.44 ± 0.02	0.99	365 ± 8	1 g/L mixture	0.41 ± 0.01	0.99	378 ± 8
1.5 g/L sinapic	0.34 ± 0.01	0.99	232 ± 6	1.5 g/L mixture	0.39 ± 0.01	0.99	292 ± 9
2 g/L sinapic	0.19 ± 0.01	0.98	44 ± 1	2 g/L mixture	0.29 ± 0.01	0.99	136 ± 4
4 g/L sinapic	0	0	0	4 g/L mixture	0.13 ± 0.01	0.99	25 ± 1

The inhibition effect of phenolic acids on methane production can be seen in Figure 3, which shows methane production rates (r) in response to phenolic acid concentrations. In the control sample of distillery stillage, the r value was 220 L/(kg VS·d). The addition of 0.5 g/L of phenolic acids (except for p-OH benzoic and sinapic acids) decreased the r value. A further increase in individual phenolic acid concentration to 1.5 or 2.0 g/L (depending on the acid type) led to an r value of 5–80 L/(kg VS·d). The inhibition constant, which is the concentration of the inhibitor (particular phenolic acid) that halves the maximum methane production rate was slightly above 1.0 g/L for p-OH benzoic and sinapic acids, slightly below 1.0 g/L for *p*-coumaric and vanillic acids, and 0.5 g/L for ferulic and syringic acids. Although hydroxycinnamic acids (including *p*-coumaric, ferulic, and sinapic acids) are considered to have greater antimicrobial activity than hydroxybenzoic acids (including p-OH benzoic, vanillic, and syringic acids) [40], the type of acid did not have any visible effect on the value of the inhibition constant in the present study. The increasing concentrations of the mixture of phenolic acids resulted in the mildest decrease of the r value, which finally reached about 39 and 3 L/(kg VS·d) at 2.0 and 4.0 g/L (Figure 3g). The inhibition constant for the mixture of acids was calculated to be 1.5 g/L.

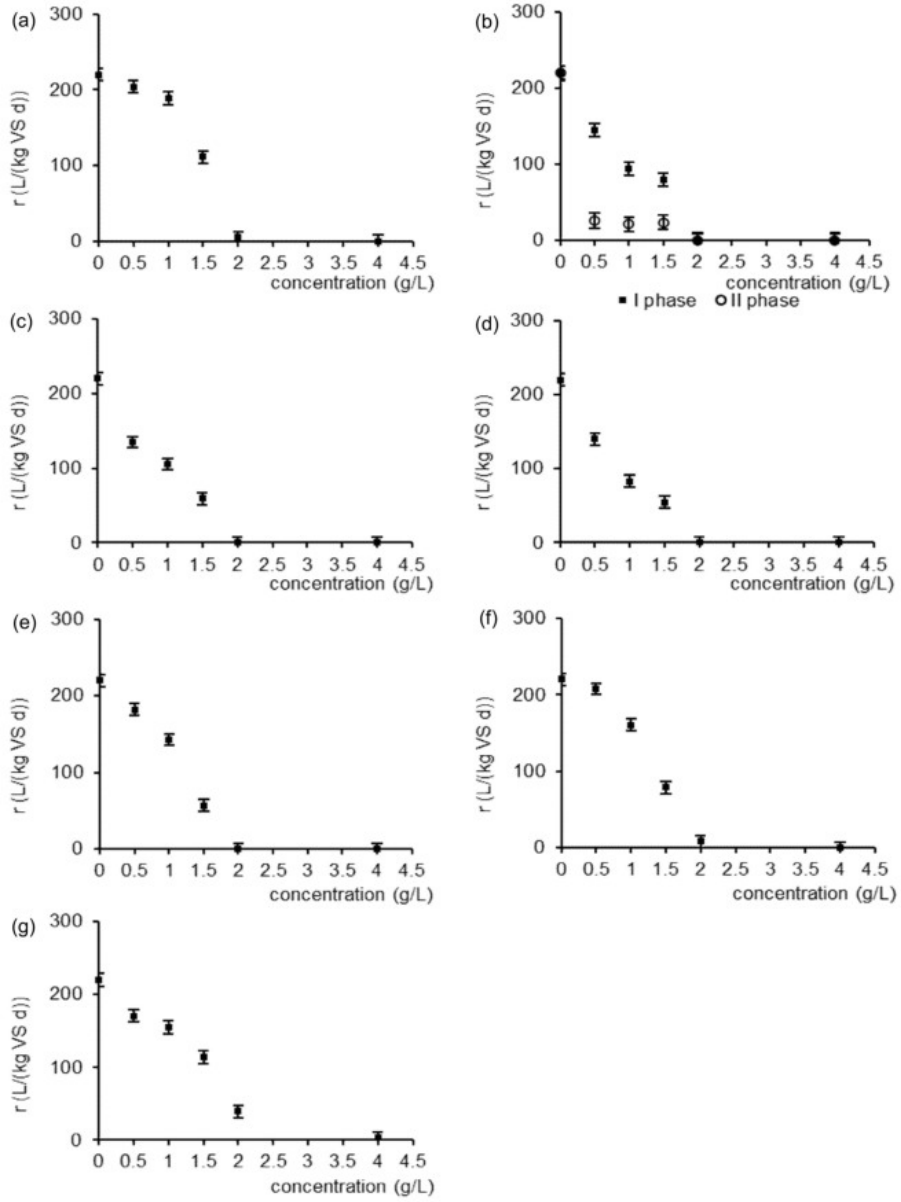


Figure 3. Methane production rates vs. initial concentrations of phenolic acids; (**a**) p-OH benzoic, (**b**) vanillic, (**c**) ferulic, (**d**) syringic, (**e**) *p*-coumaric, (**f**) sinapic, (**g**) a mixture of acids.

The toxic effect of phenolic compounds on anaerobic digestion may result from the size and polarity of their molecules as well as the substituents on the aromatic ring that modify the polarity [6,41]. Compounds with aldehyde groups or nonpolar substituents were highly toxic, while those with carboxyl groups only caused significant inhibition at high concentrations [42]. This was in agreement with the present study, which indicated that syringic and ferulic acids (containing an aldehyde group) had a stronger inhibitory effect on methane production than acids without this group (Figures 1–3). All acids with hydroxyl groups on the aromatic ring had slightly increased toxicity, but introducing further hydroxyl groups could create less-inhibitory compounds [43,44]. As a result of molecular interactions, less-toxic compounds (p-OH benzoic acids) could be formed. Aromatic compounds with more or longer alkyl substitutions (ferulic or *p*-coumaric acid) had stronger inhibitory effects than those with fewer or shorter alkyl substitutions. Thus, in the present study, p-OH benzoic acid had the weakest inhibitory effect, while ferulic acid, with the longest alkyl side chain, had the strongest inhibitory effect. It should be kept in mind that the structure of the alkyl side chain plays an important role in the antimicrobial activity of

aromatics. For example, the presence of polar functional groups on an aliphatic side chain often reduces the microbial toxicity of aromatic compounds [45]. According to Wang and Wang [46], -COOH reduces toxicity to a greater extent than OH, which in turn reduces it to a greater extent than -CH_2CH_2OH. For this reason, in the present study, sinapic acid was less toxic than ferulic, *p*-coumaric, and syringic acids, which all have similar structures. The low toxicity of sinapic acid is due to its -COOH substituent and three other substituents. The size of the structure does not affect the toxicity of the phenolic acids, because the molecular weights of ferulic, syringic, and sinapic acids are similar, even though sinapic acid had a lower methane inhibitory effect than ferulic and syringic acid. A similar effect can be seen when comparing the inhibitory effect of vanillic and *p*-coumaric acids, which also have similar molecular weights.

3.2. Characteristics of Digestate

In series 1 and 2, the conversion of organic compounds to methane did not differ significantly between the control and the experiments with phenolic acid concentrations of 0.5, 1.0, and 1.5 g/L (Table S2). However, when the phenolic acid concentrations reached 2.0 g/L, the efficiencies of TS, VS, COD, TOC, and DOC removal were noticeably reduced and further reduced at phenolic acid concentrations of 4.0 g/L.

Despite the low biodegradability of the polyphenols, they could serve as an additional carbon source for some microorganisms. Thus, at lower phenolic acid concentrations, more than 80% of the TPC were anaerobically converted, and about 20% remained in the digestate (Figure 4).

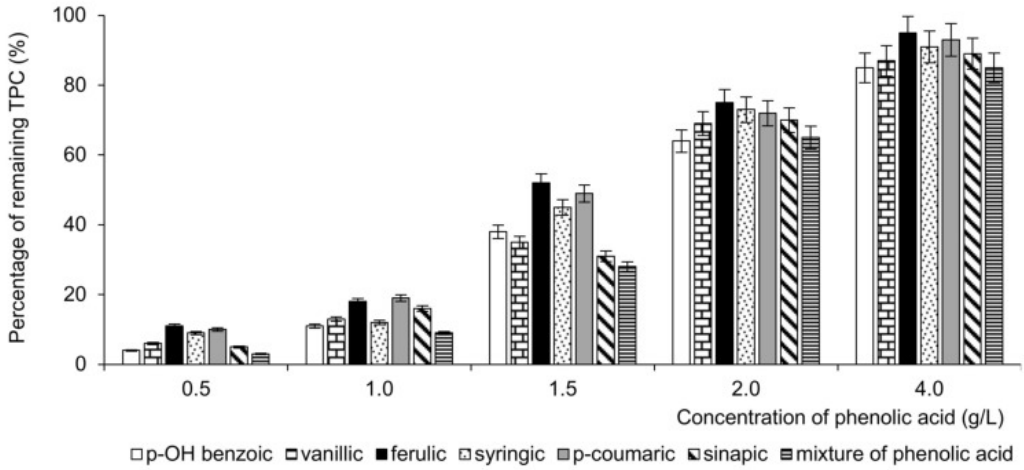


Figure 4. Percentage of undegraded TPC in digestates.

At a phenolic acid concentration of 4.0 g/L, 85–97% of TPC was not degraded and remained in the digestate. The percentage of the TPC that remained in the digestate was strongly negatively correlated with the efficiency of the TOC removal ($r_s = -0.88$), which shows that the increasing concentrations of the phenolic acids limited their use as a carbon source. For these calculations, the TPC in raw stillage without phenolic acid addition was neglected because of the low ratio between a stillage sample and inoculum in the feedstock, which was the same in all experimental variants.

The stability of the methane fermentation process was monitored based on the pH, the alkalinity, and the VFA concentrations (Table S2). Under all the experimental conditions, the pH ranged from 6.85 to 7.42; for fermentative microorganisms and methanogenic archaea, pH should fall within the ranges 5.0–8.5 and 6.5–7.8, respectively [47]. The alkalinity of the fermented stillage decreased from 186 to 127 meq/L (series 1) and from 191 to 134 meq/L (series 2) as the phenolic acid concentrations were increased from 0.5 to 4.0 g/L. Methane production was strongly positively correlated with pH (r_s = 0.81–0.94) and alkalinity (r_s = 0.86–0.97).

At the lowest concentration of phenolic acids in series 1 and 2, the VFA levels remained low due to their immediate consumption after their production. As the phenolic acid concentrations were increased, the VFA levels increased correspondingly, peaking at 3423.5 and 3075.3 mg/L in series 1 and 2, respectively, with phenolic acid concentrations of 4.0 g/L (Table S2). This increasing accumulation of VFAs may relate to the degradation of phenolic intermediates or may reflect a kinetic uncoupling between the acid producers and consumers. However, the concentrations of VFAs probably did not inhibit methanogenic activity, as they never exceed levels of 3.5–5.0 g/L when this typically occurs [48], because the VFAs were neutralized by the alkalinity, and therefore the pH was almost stable. This is more evident, because, in the present study, the VFA concentrations in the digestates obtained when distillery stillage was spiked with the phenolic acids that affected the methane production the most (vanillic, ferulic, syringic, and *p*-coumaric acids) were similar to those found for the other acids.

Despite the accumulation of VFAs, ammonium contributed to increasing the buffering capacity and keeping the pH stable [49]. At phenolic acid contents of 0.5 and 1.0 g/L, the concentrations of ammonium nitrogen, produced in anaerobic digestion during the degradation of organic nitrogen, were similar to that in the control sample (Table S2). As the phenolic acid concentration increased, the ammonium concentration increased, reaching levels of 682.7 and 625.4 mg/L in series 1 and 2, respectively, at 4.0 g/L of phenolic acids. A similar increase was observed in the case of phosphorus concentrations, which increased from about 189 mg/L in a control sample and at 0.5 g/L of phenolic acids to 368.6 mg/L at 4.0 g/L of phenolic acids. Lower concentrations of nitrogen and phosphorus in the substrate than in digestates can be explained by the fact that they were not measured in the total substrate but in the centrifuged supernatant. Thus, the substrate was characterized by the concentrations of ammonium nitrogen and phosphates, whereas the final composition of digestates (concerning N and P) resulted from the degradation of organic matter. The total nitrogen (ammonium and organic) in distillery stillage may reach 1.97 g/L [50]; organic nitrogen may account about 88% of the total [33] or above 99% of the total [27], due to high amounts of proteins in cereal-processing waste [34]. In digestates with some concentrations of phenolic acids (above 1.0–1.5 g/L), nitrogen and phosphorus concentrations were higher than in digestates from the substrate (distillery stillage) and inoculum (control sample). Due to the fact that nitrogen and phosphorus are key macronutrients for the growth of anaerobic microorganisms, this increase suggests that less biomass growth was possible, leading to a lower amount of nutrient incorporation in the new biomass cells. This indicates the inhibition of microbial growth by the phenolic acids, more so because, regardless of the type of acid, the increase in their concentration increased the concentration of nitrogen and phosphorus accumulated in the digestate. Nevertheless, the ammonium levels were still below those reported in the literature threshold concentrations that limit methanogenesis. Methane fermentation was inhibited at ammonium nitrogen concentrations higher than 2000 mg/L [51]. In other studies, ammonium nitrogen concentrations above 1000–1500 mg/L were reported as the primary cause of fermentation failure due to inhibition by free ammonia [52]. As free ammonia can easily permeate through cell membranes [52], it is the form of nitrogen that mainly inhibits methanogenesis. In the present study, due to the pH values resulting from the balance between VFAs and ammonium, the free ammonia content was low, up to 13.3 mg/L. Thus, the inhibition of biomass growth resulted in the accumulation of nutrients, and their end concentration did not affect the methane outputs. In summary, the presence of phenolic acids could have affected the growth of microorganisms, thus directly affecting methane production.

From observations of the effect of phenolic acids on bio-methanization, it is not clear which step of the anaerobic process was inhibited to a greater extent by these acids. Thus, for more insight into this question, the abundance of the most sensitive microorganisms in the fermentation chain—the methanogens—was determined in the fermented distillery stillage.

3.3. Abundance of Methanogenic Archaea

In the control sample, the abundance of Archaea was 14.5%. In the fermented stillage that was spiked with 0.5–1.5 g/L of sinapic, *p*-coumaric, vanillic, or p-OH benzoic acids or the mixture of acids, their abundance remained at a similar level (Figure 5). However, even at these low concentrations of syringic and ferulic acids, their abundance decreased to 7.6 and 5.1%, respectively. For all the phenolic acids and their mixture, increases in their concentrations to 2.0 and 4.0 g/L reduced the abundance of Archaea. This reduction was most noticeable (up to 30 times) with *p*-coumaric, syringic, and ferulic acids. Although no strong correlation between the phenolic acid concentration and the abundance of Archaea was observed, phenolic acids significantly affected the quantity and quality of biogas, which was strongly dependent on the abundance of particular Archaea.

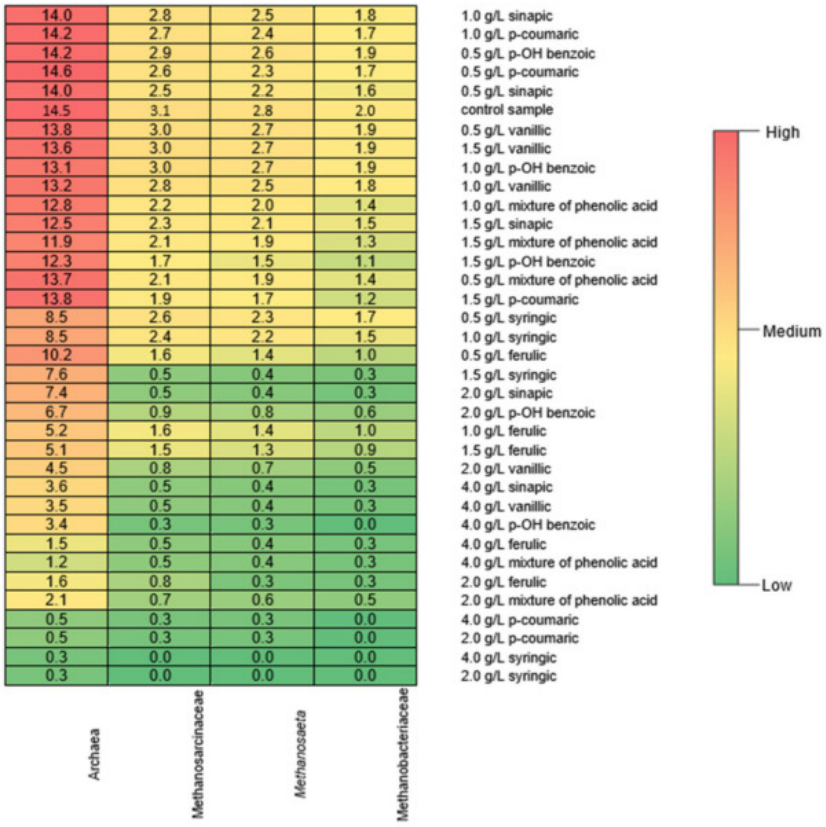


Figure 5. Heatmap of the abundance of Archaea and methanogens (%) in digestate. Grouping was based on hierarchical agglomerative clustering. The abundance of Archaea is represented by a color (light blue means lower abundance; dark blue means higher abundance). The color key is indicated in the legend in the top left of the plot along with the count of the abundance of Archaea in each titer.

In fermented distillery stillage, the acetoclastic methanogens (Methanosarcinaceae and *Methanosaeta*) and hydrogenotrophic methanogens (Methanobacteriaceae) most commonly present in fermented biomass were detected. Methanosarcinaceae can use hydrogen and carbon dioxide, methylated compounds, and acetate for methane generation [53]. They have a low affinity for acetate but have a shorter doubling time than *Methanosaeta*. Therefore, in this study, at a high concentration of VFAs, Methanosarcinaceae predominated over *Methanosaeta*. Methanosarcina is mainly responsible for methane production, because it is resistant to various stressors (a high concentration of ammonium salt or temperature variations) [54]. Therefore, in this study, methane production did not significantly decrease when 0.5–1.0 g/L of phenolic acids was present. Then, the abundances of Methanosarcinaceae, *Methanosaeta*, and Methanobacteriaceae were 3.0–1.6%, 2.7–1.4%, and 1.9–1.0% of the total microbial population, respectively. At a phenolic acid concentration of 1.5 g/L, a decrease in the abundance of these microorganisms was observed. Similarly,

however, changes in the microbial community structure were observed for 0.5 g/L of phenols [55]; the methanogenic activity was maintained with phenol concentrations increasing to 2 g/L. This was related to the increasing abundance of *Methanoculleus* at the expense of *Methanosarcina*. In the present study, a large decrease in the Archaea abundance at 2.0 and 4.0 g/L (the abundance as low as 0.5% of total microbial content) resulted in an inhibition of methane production.

The methane production by hydrogenotrophic methanogens utilizes hydrogen [56]. A decrease in the abundance of hydrogenotrophic methanogens (Methanobacteriaceae) because of biomass exposition to phenolic acids (concentrations above 1.5 g/L) in the present study could have increased the hydrogen partial pressure. As a result, the inhibition of acetogenic bacteria could have occurred, which severely disturbs the balance between VFA-producing and VFA-consuming microorganisms [57]. This caused an accumulation of VFAs (above two times higher than in the control series) in the present study. High hydrogen partial pressure could also have been the reason for the inhibition of acetoclastic methanogens. This was proved by Cazier et al. [58] by increasing the abundance of these microorganisms after CO_2 addition and could have been the reason for the complete inhibition of methane production in the present study at phenolic acid contents above 2.0 or 4.0 g/L.

In series 1 and 2, the abundance of Methanosarcinaceae and *Methanosaeta* was higher than that of Methanobacteriaceae; therefore, it can be assumed that the acetoclastic pathways dominated over the hydrogenotrophic pathways in the utilization of distillery stillage containing phenolic acids. These observations indicate that, despite shifts in the microbial community structure, the deterioration of anaerobic digestion performance was observed at phenol concentrations above 2 g/L. In contrast, at concentrations of 2 g/L, shifts in microbial community resulted in the high level of adaptability of archaeal and bacterial communities to toxic compounds [55]. Similarly, the significance of the adaptation to phenols was pointed out in studies on the digestion of olive mill wastewater; methane production increased by 280% because of biomass adaptation [32]. In the batch tests in the present study, the adaptation of methanogenic Archaea to high concentrations of phenolic acids was not observed. However, the present study suggests that the changes in the abundance of methanogens may account for early warning microbial indicators of the inhibition of anaerobic digestion by the presence of polyphenols.

3.4. Statistical Analysis

A PCA was performed to show the extent to which phenolic acids inhibit the abundance of the microorganisms participating in the bio-methanization of the distillery stillage and diminish the amount of biogas produced (Figure 6).

The PCA was shown in three plots: hydroxybenzoic acids (Poh—p-OH benzoic acid, VAN—vanillic acid, SYR—syringic acid) (Figure 6a), hydroxycinnamic acids (pCO—*p*-coumaric acid, FER—ferulic acid, SIN—sinapic acid) (Figure 6b), and a mixture of phenolic acids (Figure 6c). In each group, the variables were reduced to two main components, which together accounted for about 100% of the total variance of the studied variables. PC1 accounted for a substantially larger amount of the variance than PC2 in each case (PC1: 81.7–82.3%; PC2: 17.4–18.1%).

The position of each variable on the chart describes its relationship with the other variables. Variables that are close to each other are highly correlated. Variables on the same side of the plot are positively correlated, and variables on opposite sides are negatively correlated. Therefore, in general, the concentrations of all individual phenolic acids and the mixture of phenolic acids (shown by a blue arrow C) were negatively correlated with methanogen abundance, biogas, and methane production. In the case of the mixture of phenolic acids, the position of blue arrow C relative to the PC1 axis indicates a lower effect of the concentration on the methanogen abundance and methane production compared to the effect of the concentrations of individual phenolic acids. Considering each concentration (0.5–4.0 g/L) separately, pOH, VAN, SYR, SIN, and a mixture of phenolic acids at 2.0–4.0 g/L and pCO and FER at 1.5–4.0 g/L were grouped on the right side of the plot

close to PC1, opposite the cluster representing the pOH, VAN, SYR, SIN, and the mixture of phenolic acids at 0.5–1.5 g/L and pCO and FER at 0.5–1.0 g/L which were close to PC2.

In addition, the effect of the phenolic acids on biogas and methane production was also shown by Euclidean cluster analysis. Five clusters were formed in hydroxybenzoic acids: clusters 1 and 2 (0.5–1.0 g/L of phenolic acids), cluster 3 (1.5 g/L of phenolic acids), and clusters 4 and 5 (2.0–4.0 g/L of phenolic acids) (Figure 6a). In the case of hydroxycinnamic acids, five clusters were observed: cluster 6 (0.5 g/L of phenolic acids), cluster 7 (1.0 g/L of phenolic acids), cluster 8 (1.5 g/L of phenolic acids), and clusters 9 and 10 (2.0–4.0 g/L of phenolic acids) (Figure 6b). Using the PCA plot, it is possible to estimate the parameters with the greatest effect on biogas and methane production by their distance from the starting point (0.0) [59]. Clusters 1, 2, 3, 6, and 7 were grouped on the negative side of the plot relative to PC1. The distance from the starting point was in the following order: cluster 3 > cluster 2 > cluster 7 > cluster 6 > cluster 1. Therefore, the effect of bio-methanization was the smallest in cluster 1 compared to the other clusters. Clusters 4, 5, 8, 9, and 10 were grouped on the positive side of the plot relative to PC1. The distance from the starting point was in the following order: cluster 10 > cluster 9 > cluster 5 > cluster 4 > cluster 8. Therefore, the effect of bio-methanization was the highest in cluster 10 compared to the other clusters. The PCA analysis confirmed that hydroxycinnamic acids had a larger effect on biogas and methane production than hydroxybenzoic acids. However, it is worth noting that the farthest from the starting point were SYR, FER, and pCO at 2.0–4.0 g/L. This strongly reflects the fact that SYR, FER, and pCO, due to their structures, had the greatest inhibitory effect on anaerobic digestion, and they inhibited the growth of the microorganisms participating in the bio-methanization of the distillery stillage the most. Synergistic and antagonistic antioxidant interactions can explain why the samples with similar concentrations of total phenolic compounds displayed different activities. The results of the effect of phenolic acids on biogas production from the stillage suggest that the concentration of these bioactive compounds should be controlled. For this purpose, recovering these compounds, pretreatment techniques [4], or the addition of macro- and micronutrients that can increase the Archaea growth, thus accelerating methanogenesis [60], should be considered.

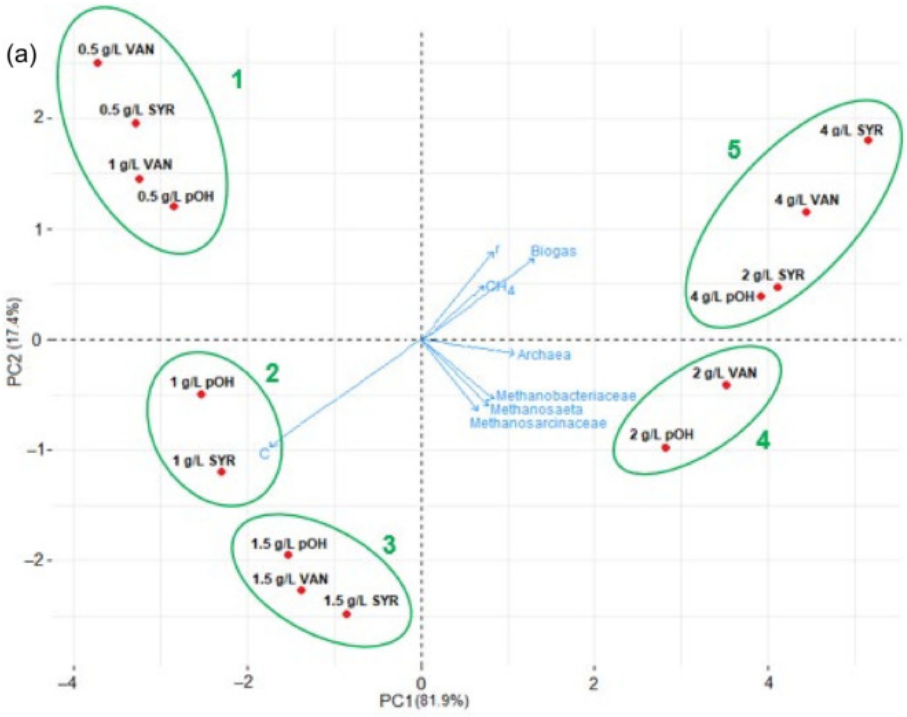


Figure 6. *Cont.*

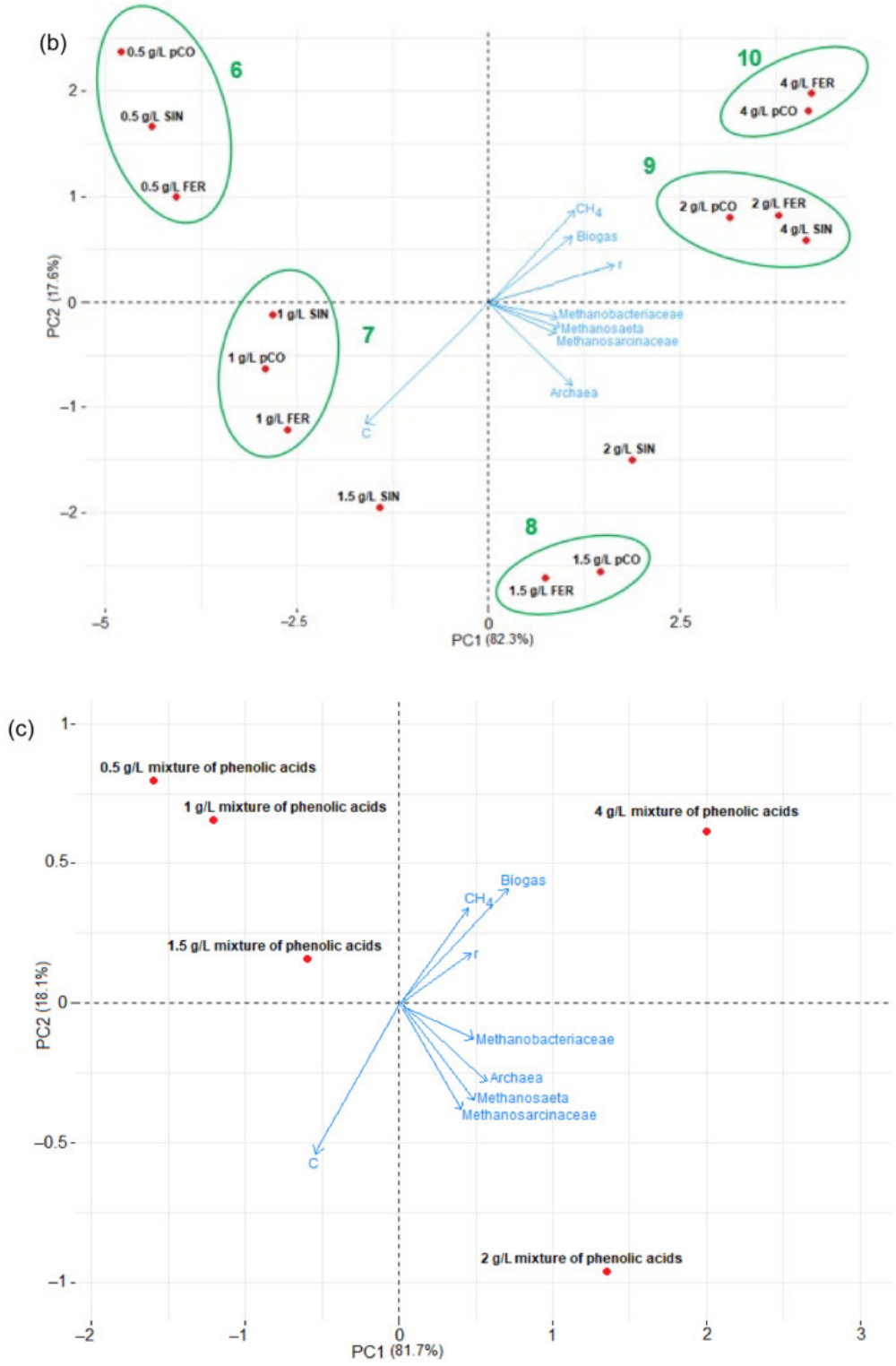


Figure 6. PCA of technological and molecular results obtained in anaerobic digestion of distillery stillage depends on the added phenolic acids; (**a**) hydroxybenzoic acids (pOH—p-OH benzoic acid, VAN—vanillic acid, SYR—syringic acid), (**b**) hydroxycinnamic acids (pCO—*p*-coumaric acid, FER—ferulic acid, SIN—sinapic acid), and (**c**) a mixture of phenolic acids. The red dots indicate the concentrations of compounds. The blue arrows represent the abundance of methanogens, the amount of methane and biogas, the rate of methane production (r), and the concentration of phenolic acids (C).

4. Conclusions

This study analyzed the interactions between the types and concentrations of phenolic acids in distillery stillage, the course of methane fermentation, and the microbial community formed during the anaerobic processing of stillage. Phenolic acids affected the microbial growth, inhibited methanogenesis, and decreased the final efficiency and rate of methane production. This effect was more noticeable when individual acids were added to the stillage than when the mixture was added. A total of 0.5–1.0 g/L of an individual phenolic acid and 1.5 g/L of the phenolic acid mixture resulted in decreasing the methane production rate by half. Vanillic, *p*-coumaric, syringic, and ferulic acids limited methane production the most; 2.0 g/L of these phenolic acids completely inhibited methane production. These results are valuable for determining the maximum allowable levels of phenolic acids in complex microbiological systems that valorize distillery stillage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15155377/s1, Figure S1: Time-course of methane production from distillery stillage with (a) p-OH benzoic, (b) vanillic, (c) ferulic, (d) syringic, (e) p-coumaric, (f) sinapic, (g) mixture of phenolic acids; Table S1: Biogas production; Table S2: Characteristics of digestate in series 1 and 2.

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Individual phenolic acids in distillery stillage inhibit its biomethanization

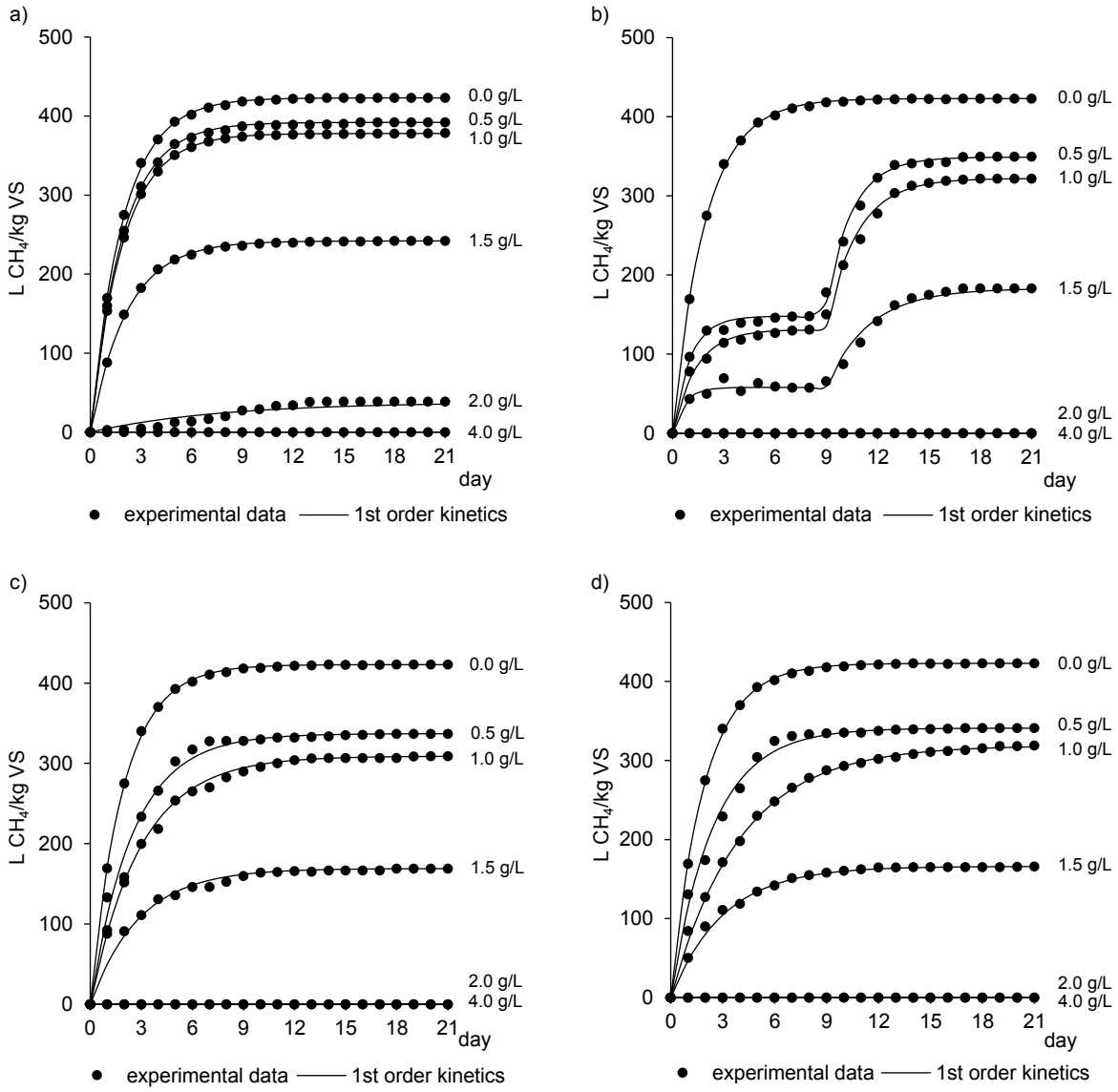
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Supplementary Materials



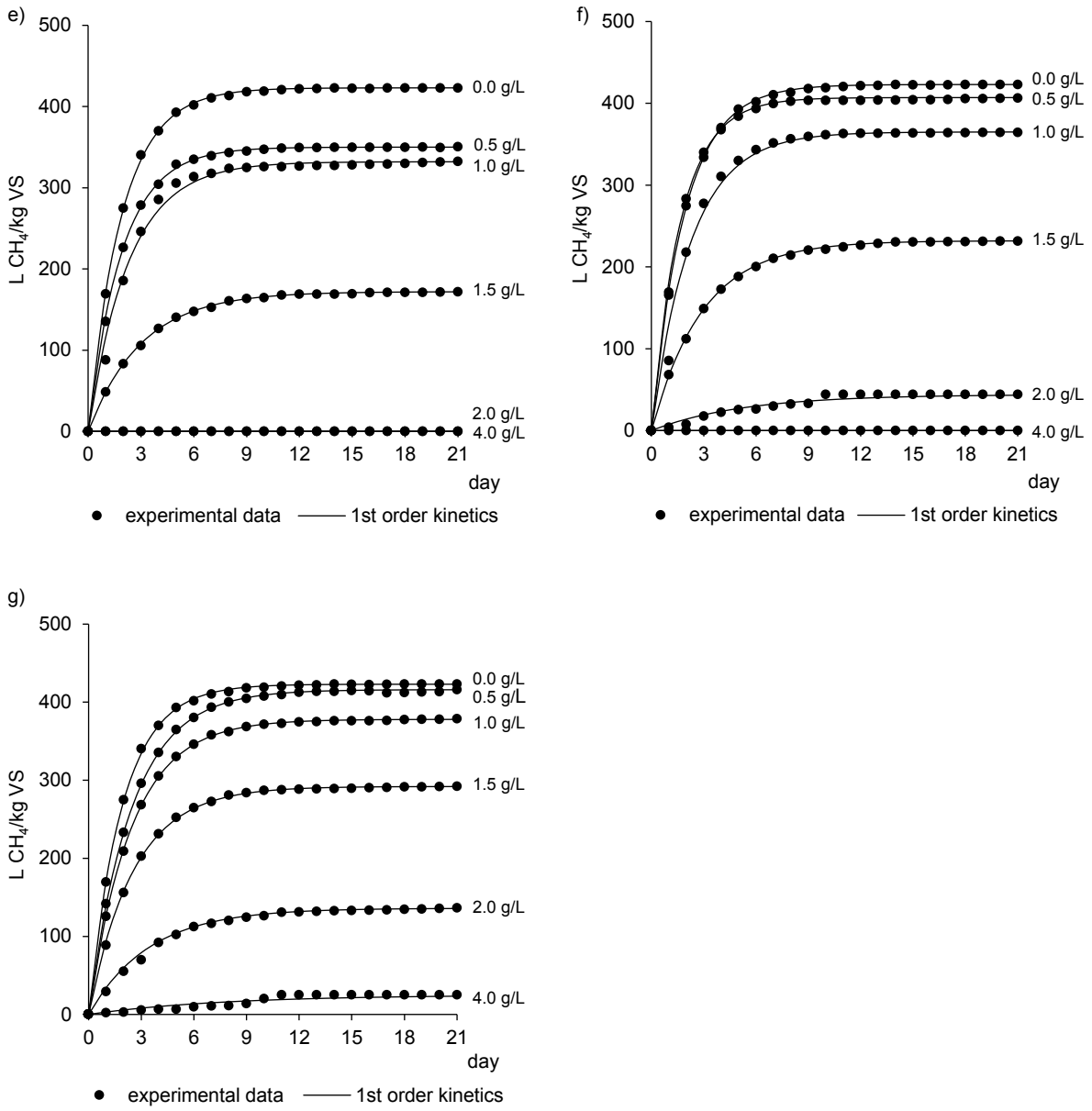


Figure 1SM. Time course of methane production from distillery stillage with a) p-OH benzoic, b) vanillic, c) ferulic, d) syringic, e) *p*-coumaric, f) sinapic, g) mixture of phenolic acids

Table 1SM. Biogas production

Type of sample	Biogas production	
	L/kg VS	L/kg Fresh Mass
control sample	544.88	43.25
0.5 g/L p-OH benzoic	510.20	40.50
1 g/L p-OH benzoic	497.87	39.52
1.5 g/L p-OH benzoic	395.33	31.38
2 g/L p-OH benzoic	70.81	5.62
4 g/L p-OH benzoic	0	0
0.5 g/L vanillic	469.42	37.26
1 g/L vanillic	445.44	35.36
1.5 g/L vanillic	303.52	24.09
2 g/L vanillic	0	0
4 g/L vanillic	0	0
0.5 g/L ferulic	457.62	36.32
1 g/L ferulic	428.74	34.03
1.5 g/L ferulic	289.11	22.95
2 g/L ferulic	0	0
4 g/L ferulic	0	0
0.5 g/L syringic	463.95	36.83
1 g/L syringic	437.45	34.72
1.5 g/L syringic	276.39	21.94
2 g/L syringic	0	0
4 g/L syringic	0	0
0.5 g/L *p*-coumaric	481.61	38.23
1 g/L *p*-coumaric	462.54	36.71
1.5 g/L *p*-coumaric	285.87	22.69
2 g/L *p*-coumaric	0	0
4 g/L *p*-coumaric	0	0
0.5 g/L sinapic	531.02	42.15
1 g/L sinapic	484.54	38.46
1.5 g/L sinapic	361.49	28.69
2 g/L sinapic	79.40	6.30
4 g/L sinapic	0	0
0.5 g/L mixture of phenolic acids	539.38	42.81
1 g/L mixture of phenolic acids	496.30	39.39
1.5 g/L mixture of phenolic acids	428.67	34.03
2 g/L mixture of phenolic acids	215.78	17.13
4 g/L mixture of phenolic acids	48.58	3.86

Table 2SM. Characteristics of digestate in series 1 and 2

Type of sample	Removal efficiency (%)					Anaerobic digestion indicators				
	TS	VS	COD	TOC	DOC	pH	Alkalinity (meq/L)	VFAs (mg/L)	Ammonium nitrogen (mg/L)	TP (mg/L)
control sample	54.1	57.2	54.7	65.1	59.5	7.42	191.5	1547.8	342.2	192.7
0.5 g/L p-OH benzoic	52.1	57.5	53.7	65.8	58.3	7.39	186.4	1623.9	302.0	195.3
1 g/L p-OH benzoic	50.3	55.4	51.5	62.9	56.4	7.38	176.0	1845.34	385.6	218.7
1.5 g/L p-OH benzoic	47.5	53.8	51.3	60.5	53.7	7.34	169.1	2037.4	435.9	257.3
2 g/L p-OH benzoic	45.8	46.5	42.9	51.2	48.9	7.35	158.8	2579.0	548.7	289.1
4 g/L p-OH benzoic	18.2	21.4	25.8	27.1	23.4	7.29	135.4	3056.2	632.6	315.1
0.5 g/L vanillic	50.3	55.9	53.7	61.4	55.3	7.42	184.2	1396.8	312.5	187.3
1 g/L vanillic	49.2	54.4	52.7	60.4	54.7	7.40	175.4	1436.9	368.2	191.2
1.5 g/L vanillic	47.2	53.2	51.0	58.1	52.3	7.36	162.2	1968.3	465.7	199.8
2 g/L vanillic	44.9	47.2	41.5	49.2	47.6	7.29	151.6	2498.7	501.4	256.4
4 g/L vanillic	16.6	20.3	27.3	25.2	21.3	7.18	134.5	3125.7	598.7	298.7
0.5 g/L ferulic	46.7	50.2	49.7	58.5	51.1	7.38	180.5	1498.7	294.7	202.4
1 g/L ferulic	46.4	51.1	49.3	55.5	51.8	7.35	165.8	1758.3	385.7	257.2
1.5 g/L ferulic	45.4	51.2	47.4	55.6	52.4	7.23	148.2	2145.1	537.3	289.5
2 g/L ferulic	37.3	43.0	40.5	48.4	44.7	7.04	135.2	2745.4	602.7	328.7
4 g/L ferulic	12.2	16.3	23.6	25.3	19.5	6.89	127.4	3423.5	658.2	368.6
0.5 g/L syringic	47.3	50.5	50.3	60.1	53.2	7.35	182.1	1475.7	285.5	189.7
1 g/L syringic	46.6	50.5	50.2	60.2	52.4	7.35	168.7	1859.3	367.0	199.0
1.5 g/L syringic	46.7	49.9	48.5	57.2	52.0	7.29	154.2	2036.4	498.4	222.8
2 g/L syringic	39.3	44.9	41.2	47.7	42.3	6.98	139.5	2746.9	572.1	243.7
4 g/L syringic	15.1	17.8	22.3	26.8	21.4	6.88	132.3	3356.3	682.7	287.5
0.5 g/L *p*-coumaric	48.2	53.3	53.3	57.3	53.5	7.32	181.5	1368.5	302.2	192.7

1 g/L *p*-coumaric	47.9	52.5	52.3	57.7	51.4	7.29	179.0	1689.6	375.2	195.4
1.5 g/L *p*-coumaric	46.7	52.4	51.3	56.4	50.5	7.21	157.4	2584.0	453.3	207.1
2 g/L *p*-coumaric	39.0	42.6	39.6	49.4	42.9	7.01	139.9	2987.7	503.5	267.0
4 g/L *p*-coumaric	13.2	18.4	23.9	25.6	19.8	6.85	128.6	3398.9	628.0	330.2
0.5 g/L sinapic	52.5	55.4	53.2	62.5	57.0	7.42	185.2	1589.6	298.8	189.6
1 g/L sinapic	51.2	54.4	53.4	59.7	55.6	7.41	168.2	1687.6	328.5	193.6
1.5 g/L sinapic	43.0	54.0	53.5	57.7	52.8	7.38	159.1	1789.6	402.9	201.3
2 g/L sinapic	42.8	45.7	41.6	49.9	47.6	7.29	142.8	2368.9	447.2	231.6
4 g/L sinapic	17.5	19.5	24.9	26.5	23.1	7.13	138.2	2975.4	587.6	304.8
0.5 g/L mixture of phenolic acids	50.4	57.3	51.0	63.4	58.2	7.36	191.7	1565.3	306.3	168.0
1 g/L mixture of phenolic acids	49.2	57.6	52.5	64.6	58.4	7.38	183.6	1668.6	357.5	181.3
1.5 g/L mixture of phenolic acids	46.6	55.4	52.5	65.7	57.5	7.31	163.4	1863.9	405.6	195.7
2 g/L mixture of phenolic acids	44.7	45.4	43.7	52.0	48.3	7.18	150.1	2038.5	501.2	278.6
4 g/L mixture of phenolic acids	18.3	21.6	27.7	29.4	23.3	7.09	134.2	3075.3	625.4	358.5

OŚWIADCZENIE WSPÓŁAUTORA O UDZIALE W PUBLIKACJI

Ja, Wioleta Mikucka, oświadczam, że mój wkład naukowy w ostateczną wersję poniższej publikacji naukowej wyniósł 65%:

Mikucka Wioleta, Zielińska Magdalena, 2022. Individual phenolic acids in distillery stillage inhibit its biomethanization. *Energies*, 15(15), 5377.

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- ☒ Udział w planowaniu badań
- ☒ Prowadzenie badań i analiz laboratoryjnych
- ☐ Konsultacja i opieka
- ☒ Udział w interpretacji wyników i wnioskowaniu
- ☒ Korekta pracy przed złożeniem do druku

10.08.2022, Wioleta Mikucka

data i podpis

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- ☒ Korekta pracy przed złożeniem do druku

10.08.2022 M. Zielińska

data i podpis

Attachment 7.

Chemosphere

The concept for the circular economy in an ethanol production plant

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research paper
Section/Category:	Environmental Chemistry
Keywords:	tetrahydrofurfuryl alcohol; biochar; recovery of phenolic acids; agro-industrial by-products; circular bioeconomy; waste-to-profit
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	Michał Binczarski
Abstract:	Solid fraction of cereal processing waste was used for preparing tetrahydrofurfuryl alcohol (THFA) and biochar (BC), which were utilized for the recovery of phenolic acids from the liquid fraction of distillery stillage (DS). The effect of THFA concentration (80 and 100%) on phenolic acid yield in ultrasound-assisted extraction was studied. The yield of phenolic acids (mainly hydroxycinnamic) in the extracts was the highest (3.76 µg/g DM) with 80% THFA. Phenolic acids from the extracts were adsorbed on 17–170 g BC/L and desorbed into the water at 40–60°C. Phenolic acid recovery was ~92% when the BC dose was 85 g/L and when desorption was performed at 50ºC. After adsorption on BC and desorption, ~95% of the antioxidant activity (AA) of phenolic acids was maintained. Similar results were obtained for commercial powdered activated carbon. These results show that THFA and BC are suitable for recovering phenolic acids in waste-to-profit management of agri-food waste.
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Highlights

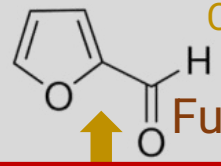
- THFA and biochar (BC) produced from cereal processing waste
- THFA and BC effective in recovering phenolic acids from distillery stillage
- Highest phenolic acid recovery with 80% THFA, 85 g BC/L and 50°C
- Predomination of hydroxycinnamic acids in phenolic acids recovered
- Similar recovery with waste-derived BC and commercial powdered activated carbon

Graphical Abstract

Biochar

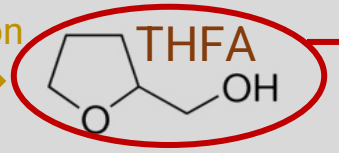


Solid residue carbonization



Furfural

Catalytic reduction



Extraction;
Adsorption/Desorption

Acid hydrolysis



Rye chaff

Rye bran



Distillery stillage



Polyphenols



in drugs and supplements



Rye

Threshing



Rye grain

Grinding

Rye flour

Grain mashing



Fermentation



Distillation/Rectification





Wheat

Threshing



Wheat grain

Grinding

Wheat flour



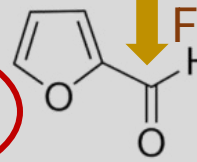
Wheat chaff

Wheat bran



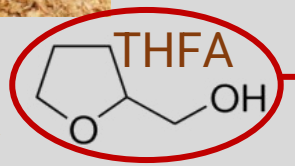
Acid hydrolysis

Solid residue carbonization

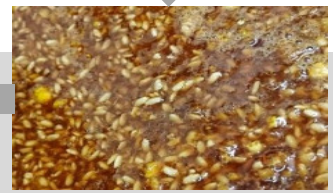


Furfural

Catalytic reduction



Distillery stillage



Extraction;
Adsorption/Desorption

Biochar



Polyphenols



in cosmetics

Manuscript File Click here to view linked References

The concept for the circular economy in an ethanol production plant

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Abstract

Solid fraction of cereal processing waste was used for preparing tetrahydrofurfuryl alcohol (THFA) and biochar (BC), which were utilized for the recovery of phenolic acids from the liquid fraction of distillery stillage (DS). The effect of THFA concentration (80 and 100%) on phenolic acid yield in ultrasound-assisted extraction was studied. The yield of phenolic acids (mainly hydroxycinnamic) in the extracts was the highest (3.76 µg/g DM) with 80% THFA. Phenolic acids from the extracts were adsorbed on 17–170 g BC/L and desorbed into the water at 40–60°C. Phenolic acid recovery was ~92% when the BC dose was 85 g/L and when desorption was performed at 50ºC. After adsorption on BC and desorption, ~95% of the antioxidant activity (AA) of phenolic acids was maintained. Similar results were obtained for commercial powdered activated carbon. These results show that THFA and BC are suitable for recovering phenolic acids in waste-to-profit management of agri-food waste.

Keywords: tetrahydrofurfuryl alcohol; biochar; recovery of phenolic acids; agro-industrial by-products; circular bioeconomy; waste-to-profit

1. Introduction

The distillery industry contributes significantly to the development of the world economy, as in 2020, about 117 billion liters of alcohol were produced (Sonnichsen, 2021), and demand for alcohol is constantly growing. Simultaneously, distilleries generate huge amounts of waste DS (up to 15-times larger in volume than the alcohol produced). The DS contains hardly-degradable organic substances, nitrogen, and dark brown pigments (Kharayat, 2012). It is promising to develop DS management in line with a circular economy. One of the steps toward achieving this goal is the recovery of valuable bioproducts from DS. Waste from cereal processing seems to be a particularly promising waste for this approach because multiple valuable products can be recovered from it (Modelska et al., 2020) that have the potential to be used synergistically, such as polyphenols, THFA and BC.

Polyphenols include phenolic acids which occur naturally as derivatives of hydroxybenzoic and hydroxycinnamic acids (Liu, 2007), have antioxidant and antimicrobial properties and thus may be used in many industries (Kiokias et al., 2020). In this context, a large emphasis is put on the search for healthy and environmentally friendly methods of phenolic acid recovery from agri-food waste. For this recovery, ultrasound-assisted extraction (UAE) is one of the most widely used methods (González-Centeno et al., 2015; Mikucka et al., 2022a).

Because the structure of phenolic compounds determines their polarity and interactions with the matrix, extractions should be optimized by selecting a solvent that is effective and does not chemically modify the solutes. To comply with the principles of green chemistry, there is a growing demand for the development of environmentally friendly solvents. The innovative solution in this area is the use of deep eutectic solvents, which are a mix of salt and hydrogen bond donors that comes from natural

metabolites (Ruesgas-Ramon et al., 2017). The most commonly used deep eutectic solvents are choline chloride-based compounds (Alam et al., 2021), which allowed, for example, obtaining the flavonoid extraction efficiencies from sea buckthorn that were significantly superior to 70% ethanol (Cui et al., 2018). Among the solvents, particularly promising are bio-solvents produced from renewable biomass sources such as energy crops, forest products, aquatic biomass, and waste materials from the sugar industry, distilleries, or mills (Torres-Valenzuela et al., 2020). During the biomass hydrolysis, pentosans are produced, and then are catalytically converted to furfural (FA). FA is a so-called platform compound, which means it is a renewable building block for products with great market potential. FA can be catalytically hydrogenated into e.g., THFA (Modelska et al., 2017). THFA has been approved by the Environmental Protection Agency as a natural and biodegradable solvent safe for the environment; however, so far, it has been used for the liquid extraction of aromatic hydrocarbons from hydrocarbon mixture (Skrollahzadeh et al., 2008). THFA may be a promising and green alternative to conventional organic solvents due to its miscibility with water, low volatility, and good thermal stability (Skrollahzadeh et al., 2008). Thus, the use of solvents derived from waste for the extraction of bioactive compounds may generate additional economic gains and positively affect the disposal of industrial by-products. However, because the extraction efficiency is affected by the affinity between solvent and a target compound and the extraction conditions, using a solvent that is unrecognized for extraction of a particular target compound needs optimization.

The phenolic acid recovery from the extracts depends on the type of solvent and the bond strength between solvent and phenolic compounds (Ruesgas-Ramon et al., 2017). Adsorption/desorption on synthetic resins was applied to recover phenolic compounds from apples (Sun et al., 2013) and grape marc (Zagklis and Paraskeva,

2015). Activated carbons have been used for the recovery of gallic, p-hydroxybenzoic and syringic acids from synthetic agroindustrial wastewater (Garcia-Araya et al., 2003) or for purification of gallic acid from *Bryophyllum* extracts (Garcia-Perez et al., 2019). Activated carbon produced by carbonization of biomass (e.g. waste coconut shell wood, plump grains, spent grains, Amerkhanova et al., 2017; Belc et al., 2019) is named BC. As the production of BC from by-products converts difficult-to-remove waste into an adsorbent for cleaning up pollutants, it is considered a doubly-effective solution in the development of sustainable technologies of waste valorization.

This work intended to take advantage of processing different streams of waste generated during cereal processing for alcohol production (the solid and liquid fractions) and reuse the products for other purposes. Kim et al. (2020) used a similarly comprehensive approach to improve the economics of the production of 2,5-furandicarboxylic acid (a precursor for renewable plastics) from lignocellulosic biomass by co-production of THFA and activated carbon. However, to the best of our knowledge, research on extracting phenolic acids from DS with "green" solvents is scarce, and there are no reports on using THFA for this purpose.

Thus, the key novelty of this study is that it combines the use of THFA and BC obtained from the solid fraction of cereal processing waste (solid fraction of DS and cereal bran) to recover phenolic acids from the liquid fraction of DS. THFA produced from furfural was used as a solvent in UAE of phenolic acids from the liquid fraction of the DS. The extracted phenolic compounds were recovered from extracts using BC. The specific objectives of the study were to determine i) the effect of THFA concentration on the extraction efficiency, the composition of phenolic acids, and the AA of the extracts; and ii) the effect of the conditions of phenolic acid adsorption/desorption (BC dose and desorption temperature) on the recovery of phenolic acids from the extracts

and their resulting AA. To examine the effect of the BC on the phenolic acid recovery, the proposed adsorption-desorption process was compared to a process that used commercially available powdered activated carbon (PAC).

2. Materials and Methods

2.1. Substrate

DS from the production of concentrated crude ethanol from cereals (mainly wheat and rye) was collected from a distillery located in north-eastern Poland. DS was characterized by: 47000±5300 mg COD/L, 4345±5 mg N_{tot}/L, 280±2 mg P_{tot}/L, and 789±3 mg CH_3COOH/L, 4.52 pH, 63.8 g dry matter (DM)/kg, and 59.5 g organic DM/kg. Rye and wheat bran were obtained from the Stoisław Mills (Polish Food Group, north-central Poland). Bran moisture did not exceed 12.5%.

2.3. Preparation of THFA

THFA was produced from the solid fraction of the cereal processing waste (solid fraction of DS and cereal bran). First, FA was produced by hydrolysis of this waste at 140°C with H_2SO_4 (> 2% by volume, 95% purity, Stanlab, Poland) as a homogenic catalyst in an autoclave (Parr Instrument Company, USA) (Table 1SM). Thereafter, the distillate was made alkaline with $CaCO_3$ (analytical grade, Stanlab, Poland) and the FA-rich fraction (~80% of FA) was isolated. Catalytic reduction of FA over homemade 0.1 g of 5%Pd/AMPS/SiO_2 catalyst into THFA was performed. Reduction of FA in water solution was made in an autoclave (Parr Instrument Company, USA) at 90°C and 20 bar hydrogen pressure (H_2, Air Products, Premium Plus, 99.999%) (Table 2SM). The catalyst and liquid substrate were placed in the reactor and the whole system was purged (20 mL/min, 15 min) with argon (Ar, 20°C, Linde 5.0, Germany), and then with

hydrogen (20°C, 15 min). Then, the hydrogen pressure was raised to 20 atm. Next, the temperature of the reaction mixture was gradually raised to 90°C. The linear temperature increase rate was 20°C/min. The reaction was continued for 2 hours.

The qualitative and quantitative composition of the reaction mixture was analyzed using a dual-channel gas chromatography system with FID and quadrupole MS detector and a SHIMADZU liquid sample feeder QP-2010 SE equipped with two SPL dispensers (GCMS-QP2010 SE). Separation of the analyte components was performed independently on two the same columns (Zebron ZB-5MSplus Capillary GC Column 30 m×0.25 mm×0.25 µm PHX-7HG-G030-11, Phenomenex, USA) connected to FID and MS detectors (sample volume 1 µL; injector temperature 200°C; furnace operation: initial temperature 50°C for 5 min, heating with a linear increase of 5°C/min to 150°C and thermostating at 150°C for 5 min). Quantitative analysis of FA, furfuryl alcohol, and THFA was performed based on the calibration curve for a concentration range of 0–10 g/L.

2.4. Preparation of BC

To produce BC, the solid fraction of the cereal processing waste (solid fraction of DS and cereal bran) was exposed to acidic hydrolysis (2% H_2SO_4, 140°C, 2 hours). The solid residual was carbonized (900°C) in a laboratory muffle oven (Czylok FCF7S, MERAZET, Poland). The oven construction enabled torrefaction and pyrolysis under anaerobic conditions. A sample of processing waste, which was started to be digested with acidic hydrolysis, was weighed before and after the process. After placing the sample in the oven, the temperature was increased to 300°C (10°C/min), then after one hour, it was increased to the desired temperature of 900°C (200°C/h). Brunauer–Emmett–Teller (BET) and Barrett-Joyner-Halenda (BJH) models were applied for the

calculation of texture properties of BC samples based on low-temperature N_2 adsorption (Table 3SM).

2.5. Extraction procedure

During UAE, 100% and 80% (v:v) THFA were used. The mixture of the DS sample (30 mL) with the solvent (30 mL) was placed in an Erlenmeyer flask and sonicated in an ultrasonic bath (InterSonic IS-5.5, Poland) (25 kHz, 560 W, 50°C, 15 min). After cooling to ambient temperature, the first part of the extract was filtered (0.45 µm filter, MCE, Chemland, Poland) under vacuum (PL 2/2 vacuum pump, Aga Labor, Poland), and phenolic acid content was measured in the supernatants. The second part of the extract was purified with activated carbons.

2.5.1. Hansen Solubility Parameter (HSP)

HSP was used to determine the affinity of the components of the extraction solvent for phenolic acids. Based on Hansen (2007), it was calculated as described in Mikucka et al. (2022b).

2.6. Adsorption/desorption

The specific surface area of BC was 273 m^2/g. The BC was ground for 1 h in a ball mill (RJM30D, Envisense, Poland) to obtain a particle size of 0.075 mm. For adsorption, the following BC doses were used: 17, 34, 85 and 170 g/L. The mixtures of the DS extracts and BC were placed in sealed stirred vessels at 6°C. After 30 min, the adsorbent was separated from the solution by microfiltration (0.45 µm). Desorption of phenolic acids was carried out by 30-min rinsing the BC with adsorbed phenolic acids with 50 mL of water (40, 50, and 60°C). After desorption, the phenolic acid solution

was microfiltered (0.45 µm) and the content of phenolic acids in the filtrate was measured with HPLC. Phenolic acid recovery by desorption was calculated:

$$Phenolic\ acid\ recovery = \frac{amount\ of\ phenolic\ acids\ desorbed}{amount\ of\ phenolic\ acid\ in\ the\ extracts} \cdot 100\%$$

The same procedure of adsorption/desorption was conducted with the use of commercial powdered activated carbon (PAC, a specific surface area of 962 m^2/g). Dubinin–Radushkevich (D–R) isotherm model was used to calculate surface area based on low-temperature N_2 adsorption. It was the commercial PAC Ercarbon GE (ERBSLOH Genseihem AG, Germany) of vegetable origin and specific adsorption capacity.

2.7. Analytical methods

2.7.1. HPLC analysis

A high-performance liquid chromatograph (Varian ProStar 325, Australia) equipped with an autosampler (MODEL 410, Australia) and a UV-Vis detector (Varian ProStar 325, Australia) was used to measure the phenolic acid content, as described by Mikucka et al. (2022a). It was measured in THFA extracts and water after desorption and expressed per gram DM of DS.

2.7.2. AA

The AA of polyphenols in the extracts was measured with ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidants power) assays (Mikucka et al., 2022a). These three spectrophotometric assays employ different reaction mechanisms based on radical scavenging activity (ABTS and DPPH) or reducing ferric iron (Fe^{3+}) to ferrous iron

200 (Fe^{2+}) (FRAP). ABTS and DPPH were expressed as micromole Trolox equivalent (TE)
201 per gram DM. FRAP was expressed as micromole $FeSO_4$ equivalent per gram DM.
202
203 *2.8. Statistical analysis*
204 Response Surface Methodology (RSM) was used to optimize the conditions for
205 adsorption/desorption of phenolic acids from DS extracts, using Statistica 13.1
206 (StatSoft). RSM used a complex design with independent variables such as water
207 temperature for desorption and activated carbon dose.
208 The analysis of Spearman's rank correlation coefficient (r_s) and PCA was
209 employed to quantify the relationships between phenolic acids, ABTS, FRAP, DPPH,
210 and operational conditions. The correlation matrix, PCA and a heatmap were made with
211 RStudio Version 1.2.1335. Statistical analyzes were performed using Statistica 13.1
212 (StatSoft) and all results were considered statistically significant at $p < 0.05$.
213
214 **3. Results and Discussion**
215 *3.1. UAE of phenolic acids from DS with THFA*
216 The UAE and its conditions were selected based on the previous study, in which
217 the UAE provided a 9-times shorter extraction time and ~1.5-times higher yield of total
218 polyphenols than conventional extraction (Mikucka et al., 2022a).
219
220 *3.1.1. Content of phenolic acids in the extracts*
221 With the use of 80% THFA, the content of total phenolic acids (TPA) in the
222 extract was 3.76±0.11 µg/g DM (Table 4SM). It was higher than the values obtained in
223 the other studies. From the same DS, UAE resulted in the TPA content of 2.67±0.04
224 µg/g DM or 2.49±0.02 µg/g DM with 80% ethanol or methanol, respectively (Mikucka

et al., 2022a), 2.12±0.03 µg/g DM with 60% acetone (Mikucka et al., 2022b), or 2.46±0.27 µg/g DM with 70% ethyl acetate (unpublished data). Although THFA has the comprehensive structure of tetrahydrofuran and methanol (Si et al., 2017), these results agree with the observations of Fu et al. (2017) that natural eutectic solvents exhibited higher efficiency of polyphenol extraction than methanol. Differences between these results obtained for the same DS can be explained by the affinity differences resulting from the different polarities of a target compound and a solvent. To select the solvent composition, the affinity of THFA and water for phenolic acids was estimated using HSP (Table 5SM). Lower values of HSP distance (R) indicate a greater thermodynamic probability that the solute and solvent will dissolve each other because adding water to the solvent makes the mixture structure less ordered, which stimulates the interaction of phenolic acids with the solvent (Zuorro et al., 2019).

As the R values were lower for 80% THFA than for 100% THFA (Fig. 1a), 80% THFA seems to be a better solvent for the extraction of phenolic acids. However, it should be taken into consideration that phenolic acids in the real samples form complex products. Thus, their affinity to solvents at various concentrations and the resulting selectivity of extraction are determined by interactions between the substrate components and bioactive compounds, which are influenced by their composition and the type of biomass from which they are recovered (Xiang and Wu, 2017). Despite this, in the present study, phenolic acid content derived with different THFA concentrations confirmed the HSP results. With 80% THFA, the yield of extracted TPA was 15% higher than that obtained with 100% THFA (Table 4SM), confirming that adding water to the solvent increases extraction efficiency. This is because water increases the diffusion of solutes to the liquid phase, which helps to weaken hydrogen bonds between the polyphenols and the matrix, thus increasing the permeability of the matrix and

improving mass transfer (Mustafa and Turner, 2011; Mikucka et al., 2022b). The addition of water could substantially reduce the viscosity (Dai et al., 2015). This is particularly important when natural solvents are used, which have a higher viscosity than conventional solvents (van Osch et al., 2017). Water reduces the surface tension, which increases the mass transfer of polyphenols, thus stimulating extraction (Chew et al., 2011). Adding water boosts the polarity of the solvent to some extent (Dai et al., 2015), which affects the solubilizing capacity of the solvents.

In the extracts, p-OH benzoic, vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids were detected. The first three acids belong to hydroxybenzoic acid derivatives, next three belong to hydroxycinnamic acid derivatives (Liu, 2007). Independently of the THFA concentration, ferulic acid contributed in almost 44% and *p*-coumaric acid in about 22% to the TPAs (Fig. 1b). The lowest share was for syringic acid (<6%). These results were expected since most cereals are rich in ferulic and *p*-coumaric acids (Shahidi and Naczk, 1995) and similar values were obtained when the same DS was investigated with the use of methanol, ethanol, acetate and ethyl acetate (Mikucka et al., 2022a,b; unpublished data). Similar ferulic acid contribution (24.0–42.1%) was reported when choline chloride:glycerol as a deep eutectic solvent was used in extraction of phenolic compounds from brewer's spent grain (López-Linares et al., 2020). The highest abundance of ferulic and *p*-coumaric acids in the present study may be attributed to their chemical structures, particularly the presence of a –CH=CH-COOH; these acids are stable and resistant to degradation due to appearing in esterified bonds (Kim et al., 2006). Additionally, high content of ferulic acid may come from the conversion of vanillic acid (Wang and Wang, 2008) which was also found in the extracts in the present study.

3.1.2. AA of the extracts

The AA of the extracts obtained with 80% THFA were 24.62±0.51 μmol TE/g DM (ABTS), 5.14±0.11 μmol TE/g DM (DPPH), and 3.43±0.06 μmol $FeSO_4$/g DM (FRAP). With 100% THFA, similar values were obtained (Table 4SM). These three tests determine various types of antioxidants and have different mechanisms of reaction, based on hydrogen atom transfer (ABTS) or single electron transfer (DPPH and FRAP). These mechanisms may occur simultaneously and taken together give complementary results of the AA (Awika et al., 2003). In the present study, the values of the ABTS were up to 5 times higher than those of the other assays because the ABTS measures the AA of a wider range of compounds. The FRAP values were the lowest because some antioxidants do not show a reducing capacity toward FRAP reagent (Huang et al., 2005). The predominance of AA shown by the ABTS and DPPH over that shown by the FRAP may indicate the predominance of phenolic compounds that are efficient at scavenging radicals and smaller content of compounds that can reduce Fe^{3+} to Fe^{2+} (Montenegro-Landivar et al., 2021).

In general, hydroxycinnamic acids have greater AA than hydroxybenzoic acids due to their chemical structure and resulting ability to donate H and subsequently stabilize radicals (Chen et al., 2020). For example, ferulic acid was reported as a great antioxidant, even at low concentrations (McCarthy et al., 2013). Ferulic and *p*-coumaric acids were considered most responsible for the AA of the DS extracts with 60% acetone (Mikucka et al., 2022b). Although in the present study ferulic acid had the highest contribution (~44%) to the TPA, there was no correlation between the AA and the phenolic acid content in the extracts. This may indicate that ferulic and *p*-coumaric acids mainly affected the AA, and differences in their high abundance did not visibly influence the AA, since the AA is dependent on polyphenol concentration to a certain

300 degree (Mouratoglou et al., 2016). This lack of correlation may also result from the
301 presence of any potential interfering species which have AA.
302
303 *3.2. Adsorption/desorption of phenolic acids from the extracts*
304 *3.2.1. Content of TPA in water after adsorption/desorption*
305 *3.2.1.1. Type and dose of activated carbon*
306 Phenolic acid recovery from BC was 60–94%. With decreasing BC dose from
307 170 to 17 g/L, TPA content in water after desorption decreased from 3.54±0.06 to
308 2.24±0.08 µg/g DM and from 3.06±0.08 to 1.92±0.05 µg/g DM from the 80 and 100%
309 THFA extracts, respectively (Fig. 2ab, Table 6SM). TPA content decreased in the
310 following order of BC dose: 170≥85>34>17 g/L and was strongly positively correlated
311 with BC dose (r_s=(0.84)–(0.87)) (Fig. 1SM). Due to similar TPA content with BC doses
312 of 170 and 85 g/L, it was concluded that the most effective TPA desorption was reached
313 when the BC dose was 85 g/L. On the other hand, the decrease in the PAC dose from
314 170 to 17 g/L had no significant impact on the TPA content. It varied from 3.70±0.06 to
315 3.27±0.04 µg/g DM and from 3.21±0.16 to 2.21±0.08 µg/g DM when processing 80 and
316 100% THFA extracts, respectively (Fig. 2cd, Table 6SM). Phenolic acid recovery from
317 PAC was 79–98%.
318 The results showed that the BC dose was of key importance for the recovery of
319 phenolic acids from DS in the adsorption/desorption. For the effective adsorption, a BC
320 dose should be much larger than a PAC dose. This was because BC had a much lower
321 specific surface than PAC, which resulted in a lower number of available activated sites
322 and thus the lower adsorption efficiency. In addition, although BC and PAC as
323 adsorbents could attach phenolic acids to their surface by physical forces, keeping the
324 compounds chemically intact, different mechanisms of adsorption occur, such as donor-

acceptor complex, hydrogen bonds, and π–π interactions (Reis et al., 2017). These interactions between phenolic acids and different adsorbents may have resulted in various adsorption efficiency.

3.2.1.2. Desorption temperature

Increasing the water temperature from 40 to 50°C increased the TPA desorption from BC (3.37±0.07 to 3.54±0.06 µg/g DM) and PAC (3.50±0.08 to 3.70±0.06 µg/g DM) from 80% THFA extracts (Fig. 2ac, Table 6SM). For 100% THFA extracts, increases in desorption ranged from 2.88±0.08 to 3.06±0.23 µg/g DM from BC and from 3.03±0.11 to 3.21±0.16 µg/g DM from PAC (Fig. 2bd, Table 6SM). A further increase in water temperature to 60°C decreased the desorption efficiency, which was lower by about 10% than at 40°C. Thus, the excessively high water temperature was not suitable for effective recovery. The highest TPA recovery was gained during desorption at 50°C, irrespective of the type of activated carbon (Fig. 2abcd). Temperatures >40°C could disturb the electrostatic interactions between phenolic acids and the adsorbent, which leads to increased solubility of polyphenols, causing the polyphenols to redissolve in the solutions. Thus, increasing kinetic energy of phenolic acids can weaken interactions with the adsorbent surface (Yu et al., 2020). On the other hand, at 60°C, degradation of thermolabile phenolic acids could occur (Calinoiu and Vodnar, 2020).

3.2.2. Individual phenolic acids after adsorption/desorption

In HPLC, high-quality peak shapes and good separation were obtained, as well as minimal baseline variability (Fig. 2SM). To illustrate the differences in the content of individual phenolic acids in water after desorption, a heat map was constructed (Fig.

3SM). After desorption from 80% THFA extracts, the contents ranged from 0.13 to 1.57 µg/g DM for BC (Fig. 3SMa), and from 0.17 to 1.64 µg/g DM for PAC (Fig. 3SMb). Phenolic acid concentrations in water after desorption from 85–170 g BC/L were higher than with the lower doses. However, the temperature had the most important role in optimizing the desorption conditions of individual phenolic acids, as was observed in the case of TPA. It can be concluded that water at 50°C can be successfully used to recover phenolic acids from DS.

In water after desorption, phenolic acid profiles were similar to those obtained in DS extracts. The content of individual phenolic acids depended on the adsorption/desorption conditions. The phenolic acid recovery from 80% THFA extracts was higher than from 100% THFA extracts. This may result from a better affinity of phenolic acids to 80% THFA and thus from a higher amount of phenolic acids in the 80% THFA extracts. The phenolic acid recovery from BC and PAC from 80% THFA extracts was 69–94% (Fig. 3a) and 92–98% (Fig. 3c), respectively. For the 100% THFA extracts, the recovery was 65–93% from BC (Fig. 3b) and 88–98% from PAC (Fig. 3d). The phenolic acid contents in water after desorption from BC and PAC were strongly negatively correlated with the THFA concentration (r_s=(-0.89)–(-0.92)) and r_s=(-0.89)–(-0.95), respectively) (Fig. 1SM)). It can be concluded that phenolic acids present in the DS can be successfully recovered using BC as a biosorbent produced from waste. It is worth noting that DS is a waste that is generated in billions of tons per year, which means that tens of kilograms of phenolic compounds can be recovered.

In the present study, 6 phenolic acids were obtained in water after desorption (Figs. 3 and 3SM) as in the DS extracts. *P*-coumaric and ferulic acids predominated in the desorbed fraction and they accounted for 15–21% and 29–41% for BC and 21–22% and 39–43% for PAC, respectively. In the desorbed fractions from both BC and PAC,

375 the smallest contribution to TPA was for vanillic acid (7–11%) and p-OH benzoic,
376 sinapic and syringic acids (5–9%). Thus, it can be concluded that the desorption process
377 does not affect the composition of the recovered phenolic acids.
378
379 *3.2.3. AA*
380 Because the structure of phenolic acids determines significantly their AA, to
381 determine whether adsorption/desorption affected the chemical changes of phenolic
382 acids, the AA after extraction and after adsorption/desorption were compared. In water
383 after desorption from BC at 50°C, the activities were 15.15–23.75 μmol of TE/g DM
384 (ABTS), 3.10–4.90 μmol of TE/g DM (DPPH) and 2.11–3.29 μmol $FeSO_4$/g DM
385 (FRAP) (Table 6SM). Fig. 4 shows the percentage of remaining AA of phenolic acids
386 after the desorption at 50°C as a function of BC and PAC and the THFA concentration
387 during the extraction relative to the AA in the extracts. The THFA concentration had the
388 greatest influence on the AA recovery. A higher percentage of remained AA was
389 observed when adsorption/desorption was used to recover phenolic acids from 80%
390 THFA extracts than from 100% THFA extracts. However, these differences decreased
391 as the amount of BC and PAC increased to 85 and 170 g/L. Phenolic acids had higher
392 AA after the adsorption/desorption with PAC than with BC (Table 6SM). The
393 percentage of AA recovered in the adsorption/desorption was 60–96% for BC (Fig. 4ab)
394 and 88–99% (Fig. 4cd) for PAC. This could have been because the AA was strongly
395 positively correlated with the phenolic acid content (r_s=(0.98)–(0.99)) (Fig. 1SM)).
396 In the present study, a high percentage of the AA of extracted phenolic acids
397 remained in the water after adsorption/desorption. This was because desorption recovers
398 only most of the active phenolic acids, leaving most of the still inactive sorbent
399 materials on the surface (Moschona et al., 2016). In this study, the content of

400 hydroxycinnamic acids accounted for up to 71% (with BC) and 74% (with PAC) of
401 TPA in water after desorption. Hydroxycinnamic acids show higher antioxidant effect
402 than their hydroxybenzoic acid counterparts due to the presence of the –CH=CH–
403 COOH connected to the benzoic ring. Therefore, in this study, hydroxycinnamic acids
404 seem to be most responsible for the AA of phenolic acids recovered from DS. The AA
405 of phenolic acids depends on the number of hydroxyl groups connected to the aromatic
406 ring. The relative positions of the –OH bound to the aromatic ring favor the antioxidant
407 and antiradical activity of phenolic acids. The number of –OH groups connected to the
408 aromatic ring in position *ortho* or *para* enhances the antioxidant and antiradical effects
409 of the phenolic acids. Substitution of the $–OCH_3$ in the position *ortho* to –OH in
410 phenolic acids strengthens AA (Sroka, 2005). Detailed studies should be carried out to
411 search if there are other compounds in the desorbed fraction that may also influence the
412 AA due to synergism.
413
414 *3.3. PCA*
415 By combining strongly correlated variables, the variables can be reduced to two
416 main components, which together accounted for 94.6% of the total variance of the
417 variables studied (Fig. 5). PC1 accounted for 85% of the variance and mainly
418 corresponded to the values of phenolic acid content, AA, type and dose of activated
419 carbon. PC2 accounted for 9.6% of the variance and corresponded to the concentration
420 of THFA and water temperature during desorption.
421 Depending on the position of each variable on the plots, the relationship between
422 the variables can be determined. Variables on the same side of the starting point (0,0)
423 are positively correlated, and variables on the opposite side are negatively correlated.
424 Therefore, all phenolic acids, AA, carbon type and dose were grouped in the cluster on

the right side, opposite to the cluster representing the concentration of THFA and water temperature during desorption from BC and PAC. The cluster variables on the right side were negatively correlated with the cluster variables on the left side. This suggests that the AA of the extracts was directly influenced by the phenolic acid content, which depended on the extraction and adsorption/desorption conditions.

Additionally, using the PCA plot, it is possible to estimate the influence of each variable based on its distance from the starting point. Thus, although the concentration of THFA affected the extraction of phenolic acids, and the temperature of water for desorption, the type, and dose of activated carbon significantly affected the adsorption/desorption of phenolic acids and the maintenance of their AA, their importance order was as follows: cTHFA>BC=PAC>mBC>tBC>tPAC>mPAC. This indicates that the solvent concentration, carbon type and dose influenced the recovery of phenolic acids the most. The impact of phenolic acids on AA decreased in the following order: FER>pCO>SIN>VAN>pOH>SYR. This indicates that hydroxycinnamic acids had the greatest effect on the AA of phenolic acids obtained after adsorption/desorption.

Moreover, the relationships between phenolic acids and the AA were shown by Euclidean cluster analysis. Three clusters were formed: cluster 1 (hydroxybenzoic acids), cluster 2 (hydroxycinnamic acids), and cluster 3 (ABTS, DPPH, and FRAP). This reflects the differences in the structure of phenolic acids and their effect on AA. Cluster 2 is further from the starting point than cluster 1, which confirms that hydroxycinnamic acids (including the dominant ferulic and *p*-coumaric acids) had a greater effect on the AA of the extracts than did hydroxybenzoic acids. The AA depended on the amount of recovered acids. Due to the predominance of hydroxycinnamic acids and simultaneous location of the ABTS and DPPH values, which were significantly higher than FRAP values, close to cluster 2, the AA may have

been mainly related to the presence of hydroxycinnamic acids. The PCA results indicated that to obtain high-quality phenolic acids during adsorption/desorption, the greatest attention should be paid to selecting the type and dose of activated carbons and water temperature during the phenolic acid desorption. However, it should also be borne in mind that due to the natural origin of the DS, the quantity and quality of phenolic acids may differ.

4. Conclusions

The synergistic use of THFA and BC (derived from cereal processing waste) was effective in recovering phenolic acids from the liquid fraction of DS with maintaining their AA. The most efficient recovery was obtained using 85 g BC/L and at 50°C in adsorption/desorption from DS extracts obtained with 80% THFA. The results provide the basis for the conclusion that the phenolic acid recovery from DS using other waste materials can increase the profitability of agri-food industry and the rate of waste utilization while reducing the environmental pollutants, which is commensurate with the circular economy principles.

Author Contributions

WM: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft Preparation, Visualization. IW: Conceptualization, Methodology, Writing – Review and Editing, Validation, Formal Analysis, Supervision. MZ: Conceptualization, Methodology, Validation, Writing – Original Draft Preparation, Writing – Review and Editing, Supervision, Funding Acquisition. KB: Methodology, Validation, Writing – Review and Editing. MB: Methodology, Validation, Investigation, Writing – Review and Editing.

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Declaration of competing interest

The authors declare no conflict of interest.

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662 ferulic acid, SIN – sinapic acid) and their AA (ABTS, FRAP, DPPH). The blue arrows
663 indicate the extraction and sorption/desorption conditions; cTHFA – concentration of
664 THFA, BC – biochar, PAC – powdered activated carbon, tBC – water temperature for
665 desorption from BC, tPAC – water temperature for desorption from PAC, mBC – a
666 mass of BC per L, mPAC – a mass of PAC per L

Figure 1

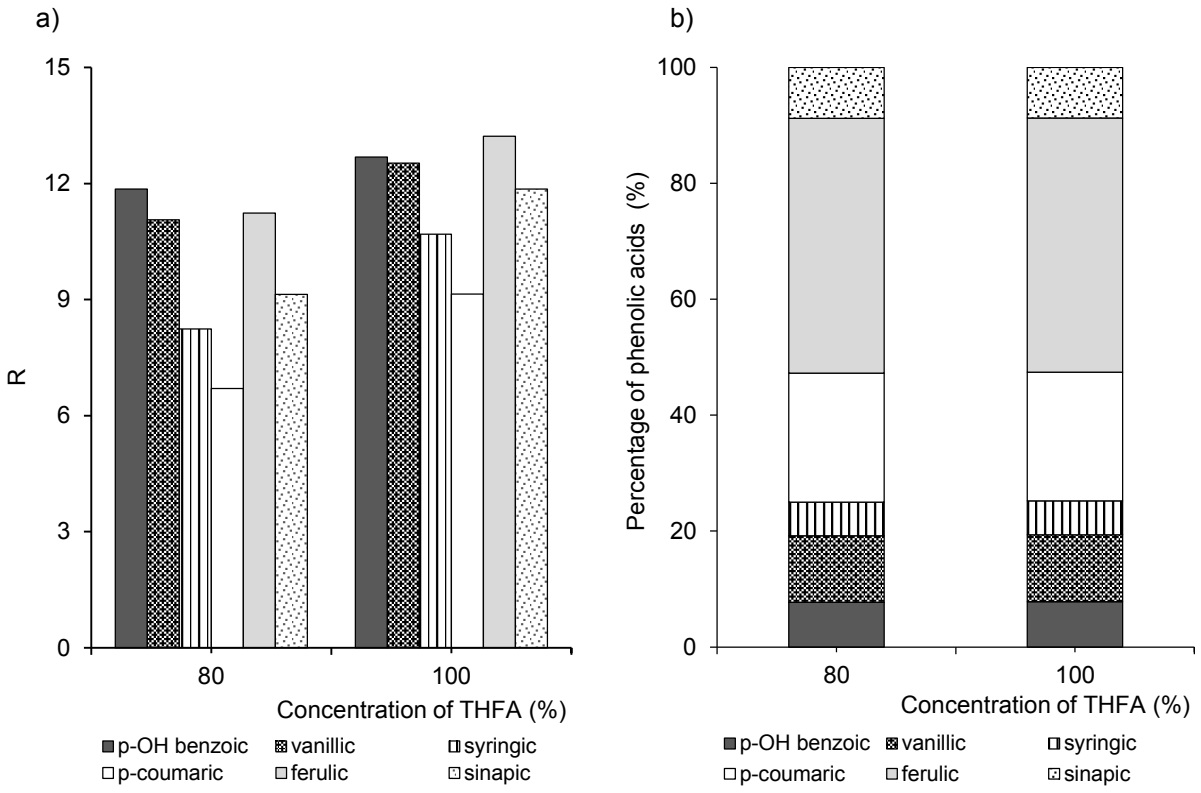

a)
15
12
9
6
3
0
R
80
100
Concentration of THFA (%)
p-OH benzoic
vanillic
syringic
p-coumaric
ferulic
sinapic
b)
100
80
60
40
20
0
Percentage of phenolic acids (%)
80
100
Concentration of THFA (%)
p-OH benzoic
vanillic
syringic
p-coumaric
ferulic
sinapic

Figure 2

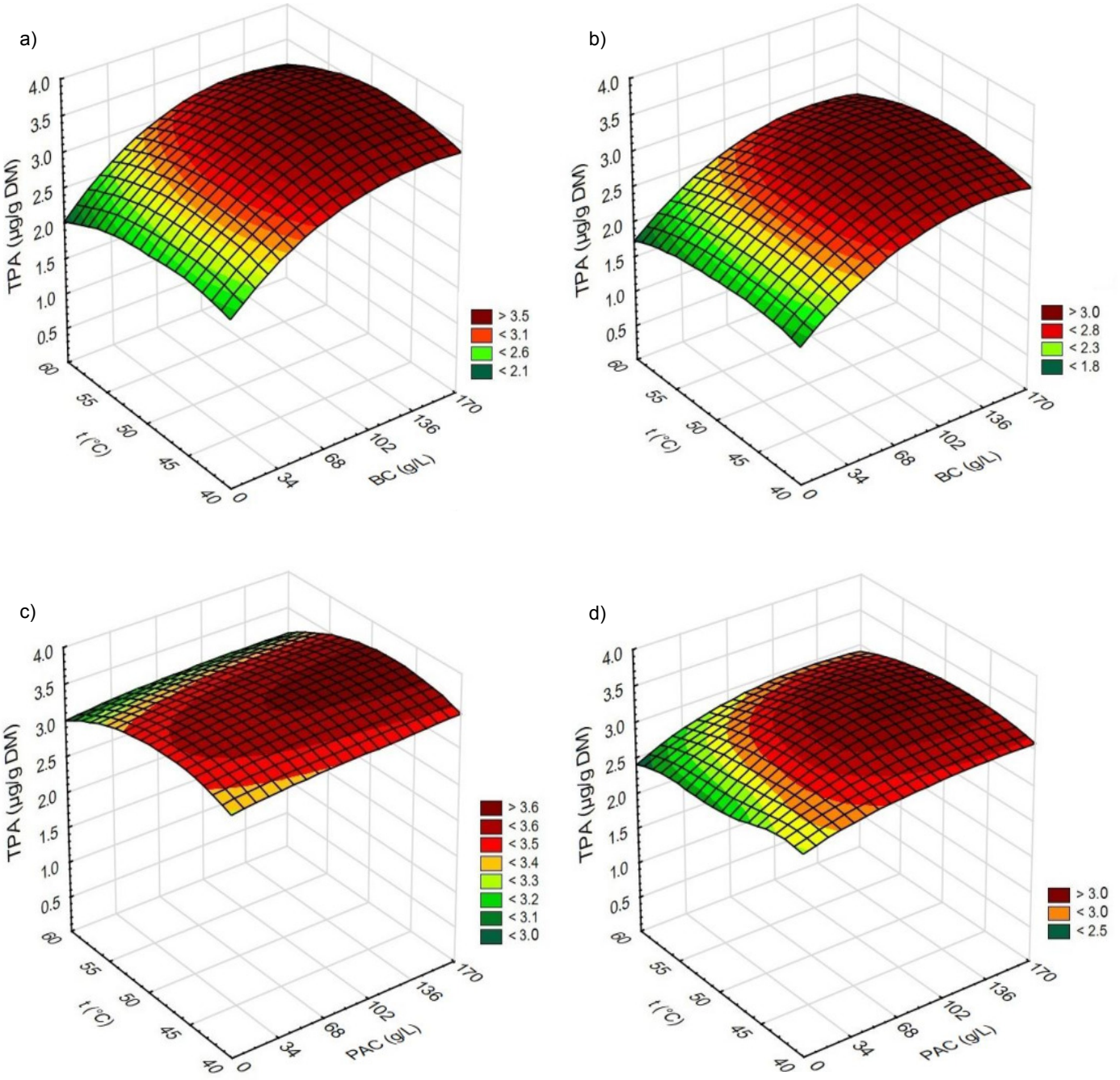

a)
TPA (µg/g DM)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
60
55
50
45
40
t (°C)
0
34
68
102
136
170
BC (g/L)
> 3.5
< 3.1
< 2.6
< 2.1
b)
TPA (µg/g DM)
t (°C)
BC (g/L)
> 3.0
< 2.8
< 2.3
< 1.8
c)
TPA (µg/g DM)
t (°C)
PAC (g/L)
> 3.6
< 3.6
< 3.5
< 3.4
< 3.3
< 3.2
< 3.1
< 3.0
d)
TPA (µg/g DM)
t (°C)
PAC (g/L)
> 3.0
< 3.0
< 2.5

Figure 3

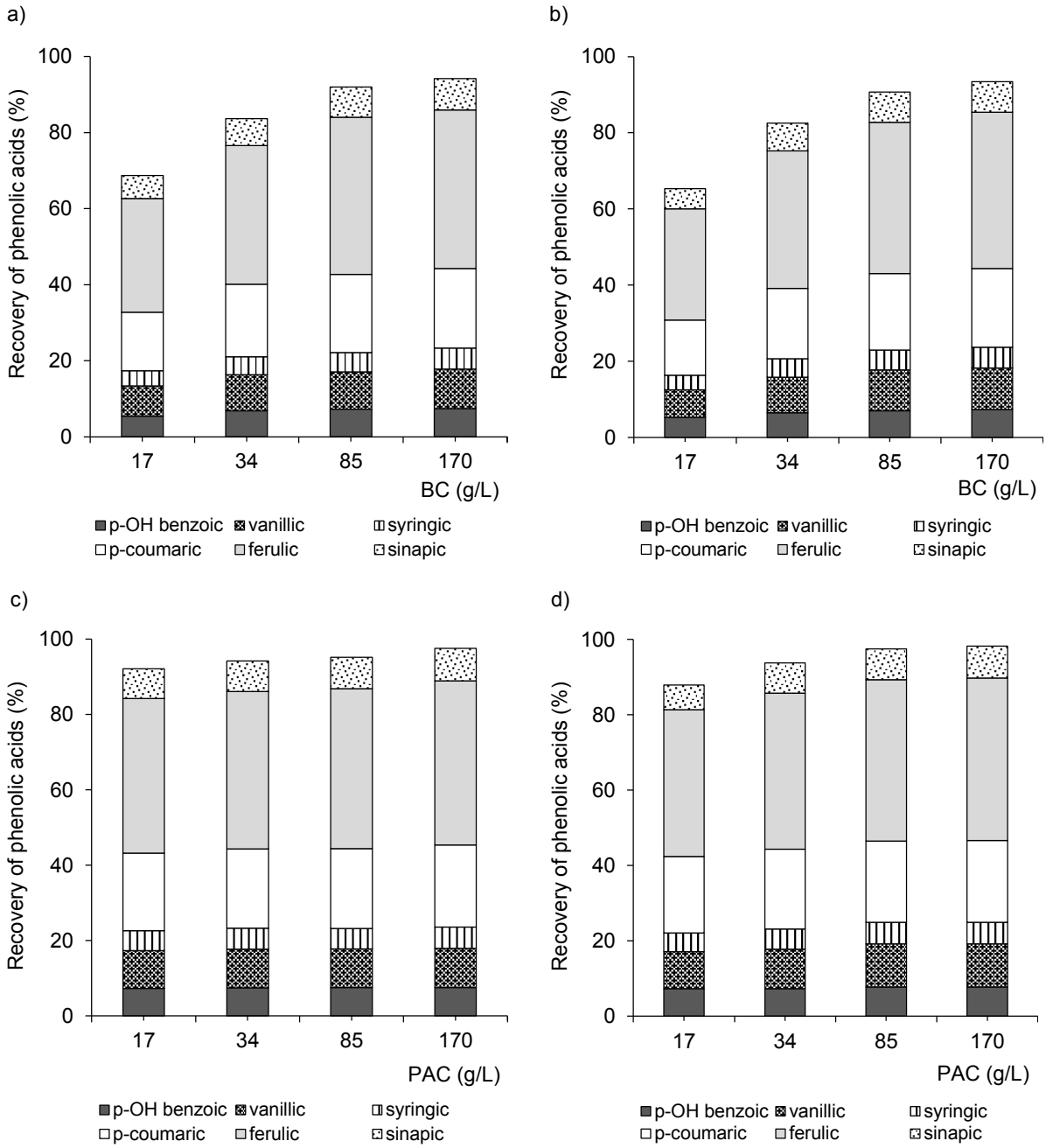

a)
b)
c)
d)
Recovery of phenolic acids (%)
0
20
40
60
80
100
17
34
85
170
BC (g/L)
PAC (g/L)
p-OH benzoic
vanillic
syringic
p-coumaric
ferulic
sinapic

Figure 4

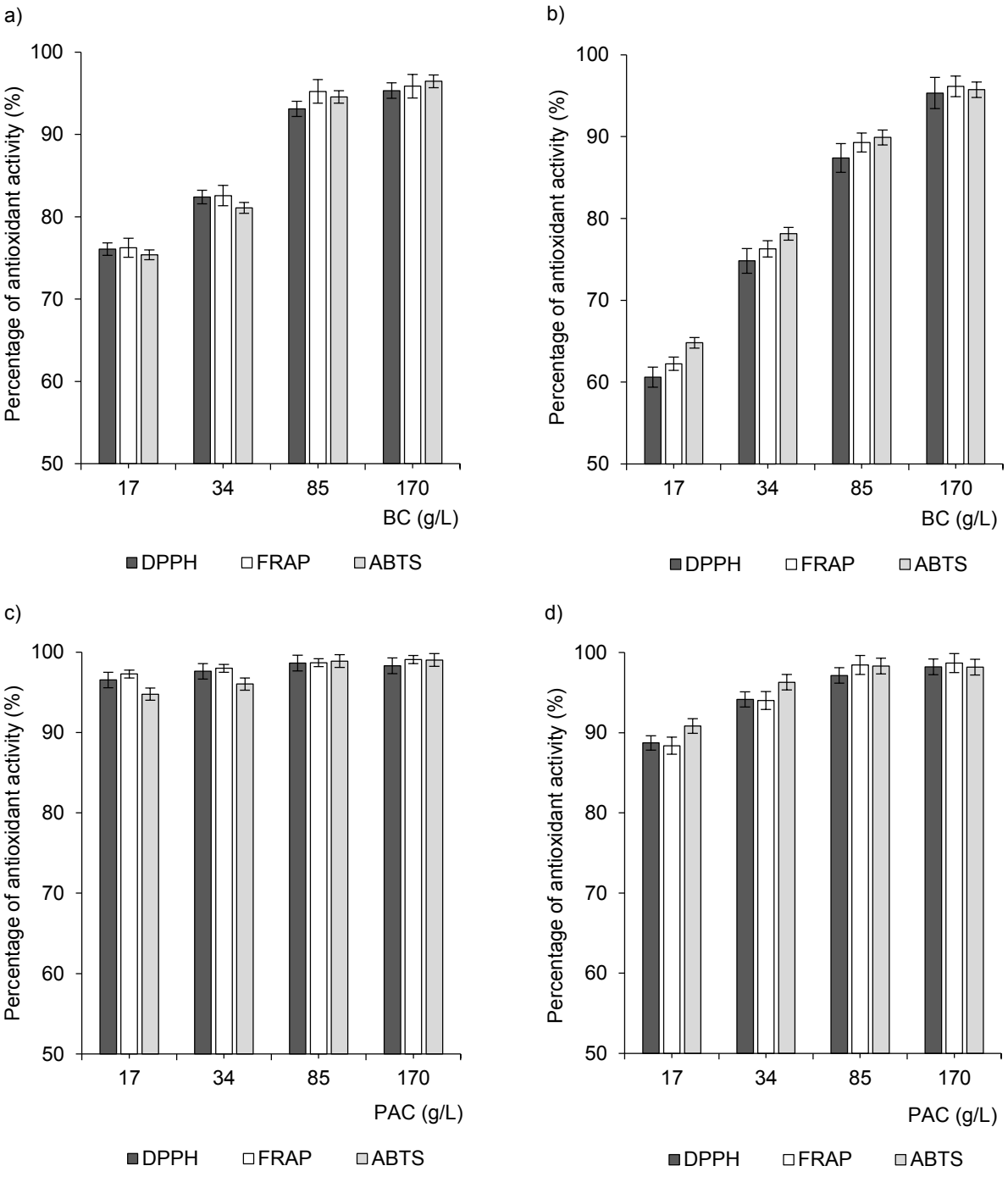
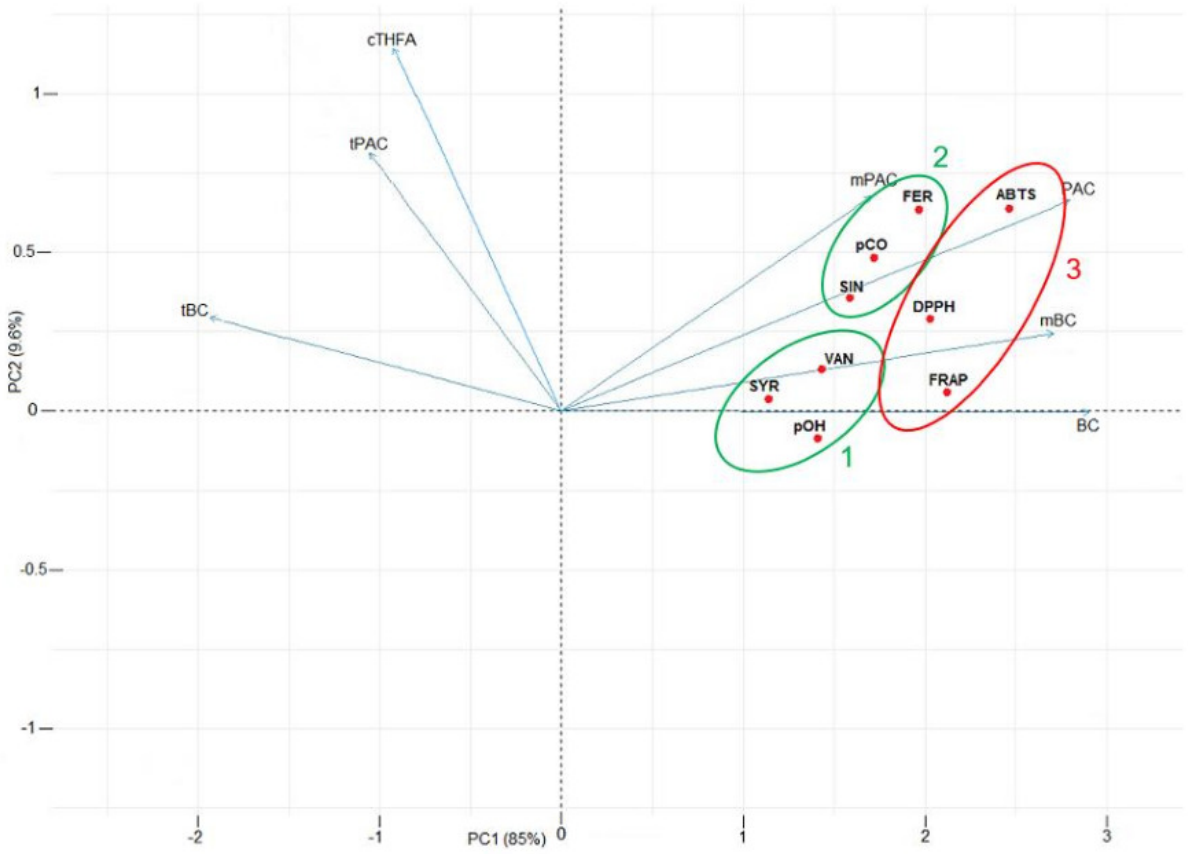

a)
b)
c)
d)
Percentage of antioxidant activity (%)
100
90
80
70
60
50
17
34
85
170
BC (g/L)
PAC (g/L)
DPPH
FRAP
ABTS

Figure 5


cTHFA
tPAC
mPAC
PAC
tBC
mBC
BC
FER
ABTS
pCO
SIN
DPPH
VAN
SYR
FRAP
pOH
1
2
3
PC2 (9.6%)
PC1 (85%)
1
0.5
0
-0.5
-1
-2
-1
0
1
2
3

The concept for the circular economy in an ethanol production plant

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Supplementary Material

Table 1SM. Yield of furfural in pressure acid hydrolysis of waste biomass from the milling industry carried out in a Parr Instrument Company 4552 pressure reactor with a capacity of 7.5 L

Raw material	C_{H2SO} (%)	T (°C)	t (h)	$m_{Raw\ material}$ (kg)/(kg DM)	V (L)	$m_{Solid\ residue}$ (kg)/(kg DM)	Loss of DM (%)	$C_{furfural}$ (g/L)	Yield of furfural per 1 kg of DM raw material (%)
Rye bran	2	140	2	0.5/0.432	5	0.421/0.033	92.36	5.754	6.75
Wheat bran	2	140	2	0.5/0.434	5	0.410/0.034	92.17	6.034	7.04
Brewing spent grains	2	140	2	0.5/0.425	5	0.406/0.042	90.12	8.010	9.56

Table 2SM. Activity and selectivity of the 5% Pd/AMPS/SiO_2 catalyst in the reduction of furfural (FA) obtained by acid hydrolysis of biomass

Substrate	Initial furfural concentration (mmol/L)	X (%)	Products (%)		
			FA	THFA	Other products
Commercial furfural	50	100	0	97.6	2.4
Furfural from spent grains	50	100	8.2	88.9	2.9
Furfural from rye bran	50	100	2.3	94.7	3.0
Furfural from wheat bran	30	100	2.1	95.9	2.1

Activation of catalysts: air drying 80°C, 12 h; reduction in H_2, at temp. 100°C, 1 h.
Reaction conditions: T = 50°C; m_{cat} = 0.1 g; $V_{furfural}$ = 25 mL; $C_{furfural}$ = 0.05 mol/L; p_{H_2} = 35 atm, t = 2 h.
X – degree of furfural conversion

Table 3SM. Texture properties of samples BC

Substrate	Specific surface area (BET) (m^2/g)	Total pore volume (BJH) (mL/g)	Average pore size (radius) (BJH) (nm)
Rye bran	118	0.139	2.73
Wheat bran	105	0.143	3.21
Brewing spent grains	83.8	0.146	3.83

Table 4SM. Phenolic acid content and antioxidant activities in distillery stillage extracts after UAE with 80 and 100% THFA

Characteristics	Unit	Distillery stillage extract	
		80% THFA	100% THFA
p-OH benzoic acid	µg/g DM	0.29±0.01	0.25±0.01
vanillic acid	µg/g DM	0.43±0.02	0.38±0.01
syringic acid	µg/g DM	0.22±0.01	0.19±0.01
p-coumaric acid	µg/g DM	0.84±0.03	0.73±0.03
ferulic acid	µg/g DM	1.65±0.04	1.43±0.04
sinapic acid	µg/g DM	0.33±0.01	0.28±0.01
TPA	µg/g DM	3.76±0.11	3.27±0.09
ABTS	µmol TE/g DM	24.62±0.51	24.92±0.07
DPPH	µmol TE/g DM	5.14±0.11	5.12±0.06
FRAP	µmol $FeSO_4$/g DM	3.43±0.06	3.39±0.02

Table 5SM. Solubility parameters contributions of dispersion energy, polarization energy, and hydrogen bond energy of solvents and phenolic acids, according to Hansen's Solubility Theory

Compound	V_m (mL/mol)	δ_D	δ_P	δ_H	δ_T
water	18.1	15.5	16.0	42.3	47.8
THFA	97.3	17.1	6.6	15.4	23.9
ferulic acid	136.8	22.7	5.7	15.6	28.1
vanillic acid	119.3	22.6	6.5	16.6	28.8
p-coumaric acid	118.1	20.4	5.6	16.0	26.5
p-OH benzoic acid	94.6	22.9	7.0	17.8	29.8
syringic acid	151.3	21.2	5.8	15.4	26.8
sinapic acid	167.3	21.6	5.2	14.7	26.6

Table 6SM. TPA content and antioxidant activities in water after desorption from BC and PAC

Concentration of THFA (%)	Type of carbon	Dose of carbon (g/L)	Temperature of water for desorption (ºC)	TPA (µg/g DM)	ABTS (µmol TE/g DM)	DPPH (µmol TE/g DM)	FRAP (µmol $FeSO_4$/g DM)
80	BC	17	40	2.47±0.05	18.08±0.23	3.66±0.04	2.43±0.05
			50	2.58±0.02	19.10±0.36	3.91±0.08	2.62±0.04
			60	2.24±0.08	16.62±0.13	3.44±0.10	2.25±0.06
		34	40	3.00±0.02	19.58±0.16	4.05±0.09	2.64±0.04
			50	3.15±0.03	20.77±0.46	4.24±0.06	2.83±0.08
			60	2.76±0.02	18.34±0.29	3.64±0.10	2.46±0.10
		85	40	3.19±0.08	22.63±0.82	4.63±0.18	3.19±0.12
			50	3.46±0.07	23.08±0.49	4.79±0.11	3.27±0.08
			60	3.04±0.05	20.01±0.26	4.09±0.12	2.79±0.12
		170	40	3.37±0.07	22.93±0.62	4.77±0.09	3.21±0.08
			50	3.54±0.06	23.75±0.14	4.90±0.12	3.29±0.07
			60	3.14±0.03	21.14±0.32	4.34±0.03	2.81±0.02
	PAC	17	40	3.27±0.04	23.46±0.85	4.80±0.19	3.31±0.13
			50	3.66±0.03	23.46±0.51	4.80±0.12	3.31±0.08
			60	3.46±0.03	23.93±0.27	4.96±0.12	3.39±0.12
		34	40	3.39±0.02	20.55±0.65	4.20±0.09	2.86±0.09
			50	3.63±0.05	24.27±0.15	5.05±0.12	3.40±0.08
			60	3.48±0.09	25.15±0.34	5.19±0.03	3.48±0.02
		85	40	3.39±0.03	22.38±0.87	4.60±0.19	2.98±0.13
			50	3.67±0.02	24.21±0.53	4.95±0.12	3.42±0.09
			60	3.48±0.02	24.70±0.28	5.12±0.12	3.50±0.12
		170	40	3.50±0.08	21.21±0.66	4.33±0.09	2.96±0.09
			50	3.70±0.06	24.53±0.15	5.10±0.12	3.44±0.08
			60	3.48±0.04	25.42±0.35	5.24±0.03	3.52±0.02
100	BC	17	40	2.06±0.05	13.79±0.73	2.85±0.13	1.92±0.09
			50	2.14±0.04	15.15±0.69	3.10±0.10	2.11±0.07
			60	1.92±0.05	12.01±0.49	2.42±0.05	1.69±0.08
		34	40	2.55±0.01	17.23±0.31	3.68±0.09	2.49±0.09
			50	2.70±0.2	18.56±0.39	3.83±0.09	2.61±0.07
			60	2.32±0.5	15.76±0.63	3.26±0.11	2.21±0.11
		85	40	2.73±0.4	21.13±0.18	4.43±0.05	2.92±0.02
			50	2.96±0.06	22.40±0.09	4.47±0.01	3.02±0.03
			60	2.60±0.3	19.54±0.47	3.95±0.03	2.70±0.08
		170	40	2.88±0.08	22.41±0.08	4.62±0.10	3.05±0.02
			50	3.06±0.23	23.55±0.27	4.88±0.04	3.21±0.04
			60	2.71±0.02	20.26±0.34	4.18±0.02	2.83±0.04
	PAC	17	40	2.42±0.04	18.89±0.90	3.91±0.11	2.63±0.03
			50	2.51±0.05	18.63±0.85	3.82±0.09	2.59±0.07
			60	2.21±0.08	15.74±0.60	3.17±0.10	2.22±0.01
		34	40	3.01±0.01	20.34±0.37	4.34±0.11	3.94±0.01
			50	2.79±0.05	21.79±0.46	4.52±0.08	3.08±0.05
			60	2.74±0.01	18.60±0.74	3.84±0.13	2.61±0.07
		85	40	3.02±0.03	23.30±0.20	4.89±0.03	3.22±0.09
			50	3.11±0.02	24.69±0.10	4.93±0.03	3.33±0.04
			60	2.86±0.03	21.55±0.52	4.36±0.09	2.98±0.06
		170	40	3.03±0.11	23.53±0.08	4.85±0.03	3.20±0.10
			50	3.21±0.16	24.26±0.29	5.03±0.04	3.30±0.04
			60	2.90±0.04	21.27±0.36	4.39±0.04	2.97±0.08

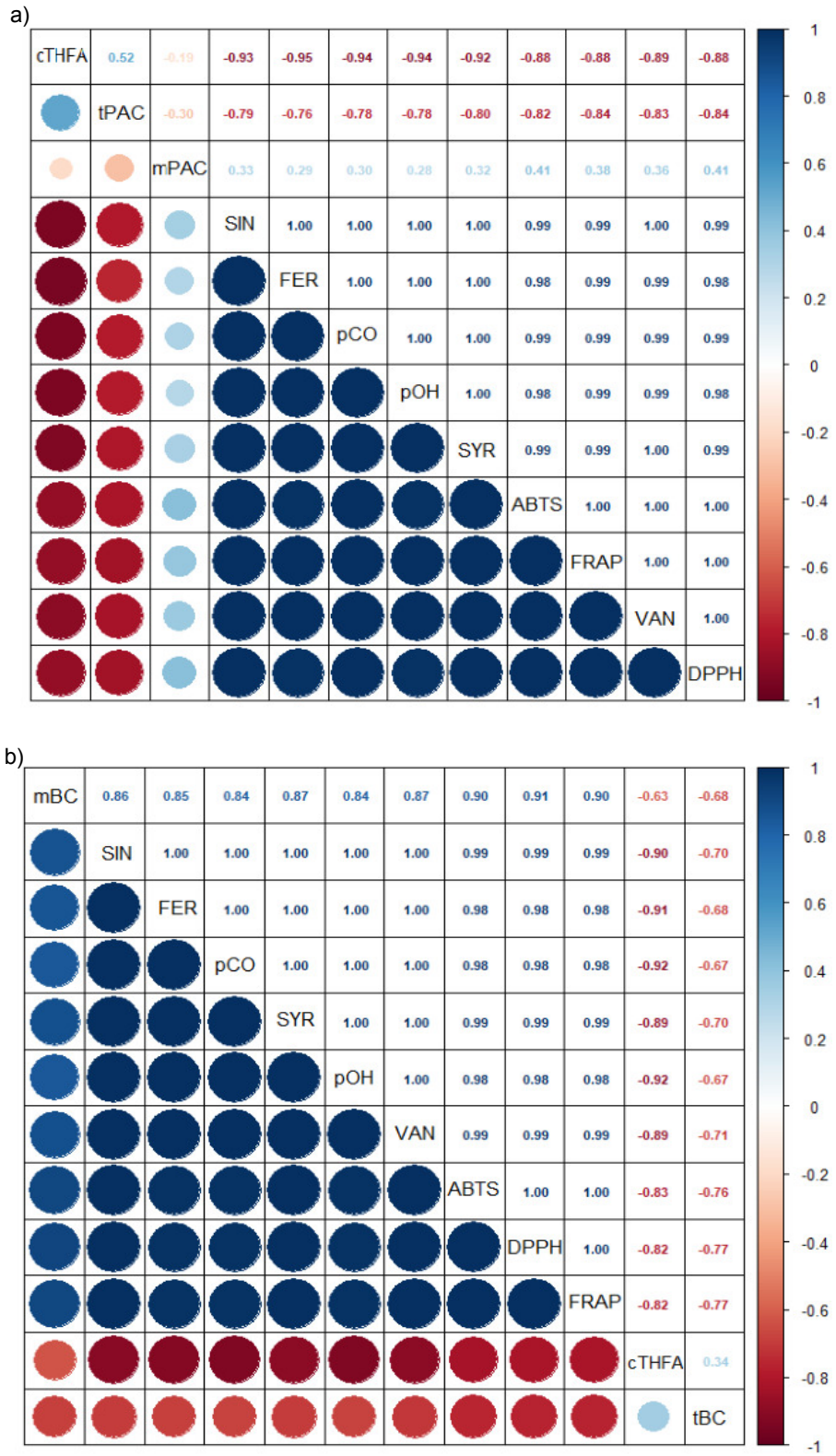


Fig. 1SM. Correlations between phenolic acid content, antioxidant activities and adsorption/desorption conditions obtained with a) BC and b) PAC. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circles are proportional to the correlation coefficients. Values of Spearman's rank correlation coefficient above 0.6 are described as a strong correlation; pOH – p-OH benzoic acid, VAN – vanillic acid, SYR – syringic acid, pCO – *p*-coumaric acid, FER – ferulic acid, SIN – sinapic acid, cTHFA – concentration of THFA, BC – biochar, PAC – powdered activated carbon, tBC – water temperature for desorption from BC, tPAC – water temperature for desorption from PAC, mBC – mass of BC per liter, mPAC – mass of PAC per liter

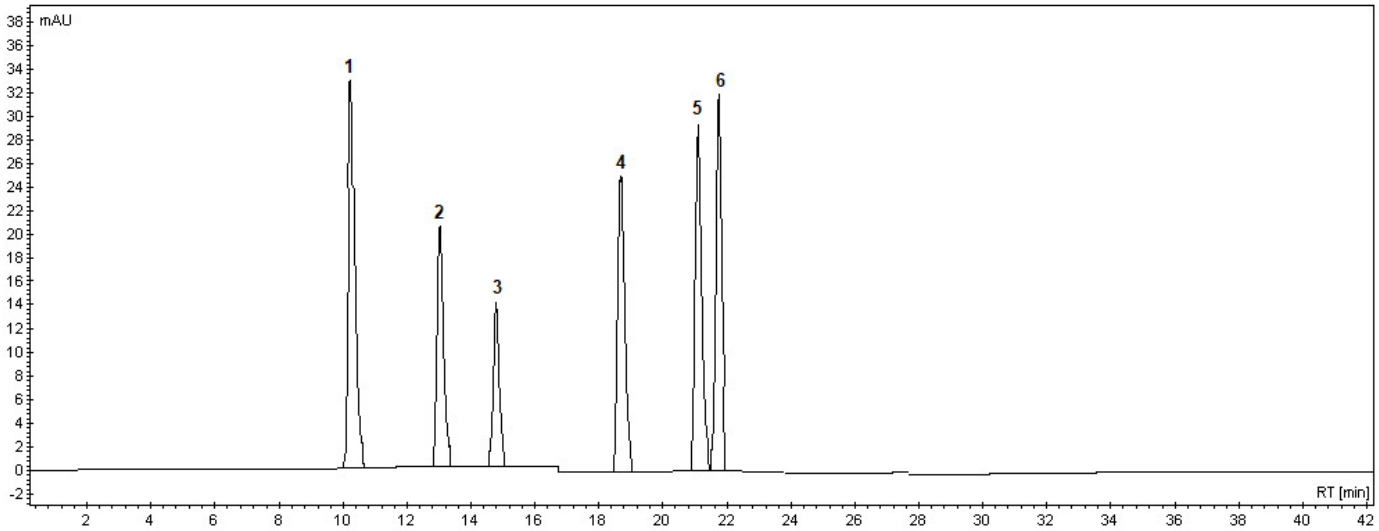


Fig. 2SM. HPLC chromatogram of phenolic acid standards at 260 nm (1 – p-OH benzoic, 2 – vanillic and 3 – syringic acid), and at 320 nm (4 – *p*-coumaric, 5 – ferulic and 6 – sinapic acid)

a)

	p-OH benzoic	vanillic	syringic	p-coumaric	ferulic	sinapic
80% 17g/L 40°C	0.190	0.286	0.143	0.553	1.087	0.216
80% 17g/L 50°C	0.203	0.300	0.150	0.579	1.125	0.226
80% 17g/L 60°C	0.176	0.261	0.130	0.512	0.968	0.196
80% 34g/L 40°C	0.240	0.332	0.174	0.687	1.309	0.255
80% 34g/L 50°C	0.255	0.358	0.179	0.716	1.373	0.265
80% 34g/L 60°C	0.223	0.309	0.158	0.644	1.190	0.234
80% 85g/L 40°C	0.251	0.343	0.182	0.724	1.420	0.269
80% 85g/L 50°C	0.272	0.369	0.192	0.774	1.555	0.297
80% 85g/L 60°C	0.241	0.334	0.175	0.680	1.353	0.259
80% 170g/L 40°C	0.261	0.370	0.196	0.751	1.499	0.292
80% 170g/L 50°C	0.278	0.392	0.208	0.786	1.567	0.312
80% 170g/L 60°C	0.244	0.343	0.183	0.698	1.400	0.271
100% 17g/L 40°C	0.166	0.225	0.120	0.456	0.924	0.165
100% 17g/L 50°C	0.171	0.236	0.125	0.475	0.956	0.173
100% 17g/L 60°C	0.153	0.210	0.112	0.418	0.870	0.153
100% 34g/L 40°C	0.198	0.297	0.150	0.562	1.118	0.224
100% 34g/L 50°C	0.209	0.308	0.157	0.605	1.182	0.239
100% 34g/L 60°C	0.181	0.255	0.136	0.530	1.012	0.206
100% 85g/L 40°C	0.216	0.320	0.165	0.613	1.178	0.243
100% 85g/L 50°C	0.228	0.350	0.171	0.658	1.299	0.260
100% 85g/L 60°C	0.200	0.296	0.152	0.589	1.139	0.221
100% 170g/L 40°C	0.223	0.329	0.168	0.641	1.263	0.252
100% 170g/L 50°C	0.240	0.356	0.179	0.675	1.343	0.262
100% 170g/L 60°C	0.212	0.317	0.157	0.603	1.187	0.232

b)

	p-OH benzoic	vanillic	syringic	p-coumaric	ferulic	sinapic
80% 17g/L 40°C	0.257	0.352	0.187	0.743	1.457	0.277
80% 17g/L 50°C	0.274	0.379	0.197	0.774	1.545	0.294
80% 17g/L 60°C	0.238	0.330	0.173	0.672	1.337	0.256
80% 34g/L 40°C	0.266	0.377	0.200	0.765	1.526	0.298
80% 34g/L 50°C	0.280	0.396	0.209	0.802	1.590	0.314
80% 34g/L 60°C	0.241	0.339	0.181	0.690	1.483	0.278
80% 85g/L 40°C	0.266	0.364	0.193	0.767	1.504	0.285
80% 85g/L 50°C	0.282	0.391	0.204	0.799	1.594	0.304
80% 85g/L 60°C	0.245	0.341	0.179	0.693	1.379	0.264
80% 170g/L 40°C	0.269	0.389	0.209	0.783	1.543	0.311
80% 170g/L 50°C	0.283	0.426	0.212	0.821	1.637	0.324
80% 170g/L 60°C	0.254	0.362	0.193	0.757	1.478	0.291
100% 17g/L 40°C	0.207	0.309	0.154	0.625	1.265	0.207
100% 17g/L 50°C	0.238	0.320	0.164	0.664	1.275	0.213
100% 17g/L 60°C	0.201	0.296	0.146	0.607	1.139	0.201
100% 34g/L 40°C	0.214	0.321	0.167	0.643	1.320	0.244
100% 34g/L 50°C	0.237	0.343	0.175	0.703	1.354	0.262
100% 34g/L 60°C	0.204	0.301	0.151	0.625	1.194	0.213
100% 85g/L 40°C	0.228	0.333	0.172	0.656	1.339	0.258
100% 85g/L 50°C	0.251	0.375	0.188	0.684	1.401	0.267
100% 85g/L 60°C	0.209	0.306	0.157	0.630	1.256	0.224
100% 170g/L 40°C	0.233	0.342	0.180	0.664	1.375	0.266
100% 170g/L 50°C	0.252	0.374	0.188	0.710	1.412	0.276
100% 170g/L 60°C	0.227	0.339	0.168	0.646	1.271	0.249

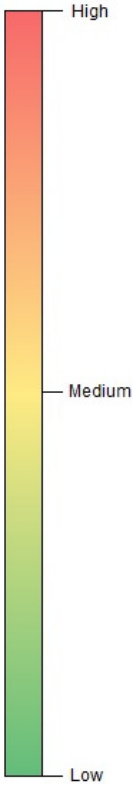


Fig. 3SM. Heatmap of the contents of individual phenolic acids (µg/g DM) in water after desorption from a) BC and b) PAC. In the series descriptions, percentage concentrations of THFA, BC and PAC doses and water temperature for desorption are given. The content of individual phenolic acids is represented by a color (red indicates a high content; green indicates a low content)

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